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METABOLIC STUDIES ON LIPID AND PROTEIN IN GERMINATING AND DEVELOPING FLAXSEED, *LINUM USITATISSIMUM* (L.)

by

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The undersigned hereby certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Metabolic Studies on Lipid and Protein in Germinating and Developing Flaxseed, *Linum usitatissimum* (L.)," submitted by Rueben E. Huber, B.Sc. (Agric.), in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The metabolism of lipid and protein fractions in developing and germinating flaxseed was investigated. Fatty acid concentration in early development stages of field and greenhouse grown plants showed similar increases, but the fatty acid composition of the mature seed appeared to be influenced by environment. The pattern of fatty acid interconversions during lipid synthesis favored the view that more unsaturated acids are formed from less unsaturated ones. Studies at later stages of seed development indicated that the lipid was in a state of dynamic equilibrium. The rapid drop in oil content during the first few days of germination indicated operation of the glyoxylate cycle. However, the relative proportion of the various fatty acids during germination remained constant, suggesting that fatty acids were being broken down at a rate proportional to the amount originally present. During flaxseed development protein content increased steadily. Until four weeks after blooming each amino acid increased, but the free amino acid portion and amide nitrogen decreased. Subsequently all remained at a constant level. The radioisotope studies gave no clear indication of amino acid interconversions. Although the protein and total amino acid composition of germinating flaxseed remained constant, the free amino acid content rose, indicating protein breakdown.

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INTRODUCTION

Lipid and protein, the two major components of mature flaxseed, account for more than half of the total seed weight. Like many other oilseeds, flax is therefore suitable for studies on the metabolism of these two components. Recently Stumpf and Bradbeer (11) reviewed the literature pertaining to changes in the fatty acid composition of oilseeds. However, none of the studies, including more recent ones, have dealt with changes in both the fatty acid and amino acid composition of developing and germinating seed. More detailed information of this nature was considered essential. Accordingly, an attempt was made in this investigation 1) to follow changes in the lipid and protein fractions of flaxseed by determining the fatty acid and amino acid composition at different stages of development and germination, and 2) to determine the pattern of fatty acid and amino acid synthesis using C^{14}-tracer methods.

LITERATURE REVIEW

I. Lipid Metabolism

"Lipid" is a term used to describe a group of fats and fat-like substances which make up a major class of tissue components and an important foodstuff. Generally included in the term "lipid" are neutral fats, fatty acids, waxes and sterols, phospholipids, cerebrosides, lipoproteins, carotenoids and some vitamins. This review of literature is restricted to include mainly a discussion of fatty acids and neutral oils. A fatty acid is a hydrocarbon chain compound with a carboxyl acid group at one end. The hydrocarbon chain may be saturated or unsaturated. Neutral fats are usually mixtures of heterogeneous triglyceride molecules which are glycerol molecules esterified at the three hydroxyl groups with two or three different fatty acids.

All higher plants contain some proportion of lipids which they synthesize. The role the lipids play varies with the plant. In most plants the lipids play a structural role, mainly as lipoprotein. In other plants they serve as a storage product and in this case the plant is known as an oilcrop.

In the years since World War II there has been rapid growth in the field of lipid biochemistry in higher plants. This has been mainly due to the development of gas chromatography, the availability of radioactive substrates and the general increased interest in lipid research by biochemists.

Fatty acid and lipid synthesis

Fat synthesis in higher plants occurs in the developing seed from precursors such as carbohydrate breakdown products (1, 2, 3, 4). The

main breakdown product of carbohydrate in the plant is acetyl Co-enzyme A (CoA). It is estimated that approximately 3/4 of all the carbon in a typical plant was at one time or another in the form of acetyl CoA (5). It is a unit of central importance in metabolic processes such as respiration, amino acid formation, fat breakdown, etc. and is a link in the plant between the TCA cycle and glycolysis.

Until recently it was thought that acetyl CoA simply condensed with itself to give long chain fatty acids. This seemed quite logical because most plant and animal fatty acids had an even number of carbons.

Newcombe and Stumpf (6) in 1952 studied the synthesis of long chain fatty acids using slices of germinating and developing peanut cotyledons. The slices metabolized a host of substrates which were chosen as potential fatty acid precursors. The results were as follows with values given as the percentage of initial activity transferred to long chain fatty acids:

Acetate 1, 2 -C^{14}	56%
Glucose and fructose	6%
Butyrate-1-C^{14}, caproate-1-C^{14}, valerate-1-C^{14}	each 1 to 3%
Formate, pyruvate, succinate	each $<1\%$.

The very low utilization of C_1-formate was taken as evidence that CO_2 fixation was not directly involved in fatty acid synthesis. They found that the incorporation of acetate was surprisingly similar in the cotyledons during germination and development, indicating that the fatty acids are in dynamic equilibrium with their acetate precursors. Several other workers (7, 8, 9) found a host of labelled substrates, mainly carbohydrate, were incorporated into various tissue lipids.

In 1956 Gibble and Kurtz (10) reported multiple β-condensation of acetate. They supplied acetate-1-C^{14} as substrate for developing flax fruits and found the odd numbered carbons of the long chain fatty acids were most highly labelled.

Considerable work has been carried out on the role of cofactors in fatty acid synthesis. Squires and Stumpf (11) using acetone powders prepared from avocado and Wakil et al (12) for chicken liver fractions found a requirement for TPNH,* bicarbonate, and ATP when acetyl CoA was the substrate. Squires, Stumpf and Schmid (13) demonstrated an increase in lipid synthesis of avocado with bicarbonate addition. Brady and Gurin (14) in 1952 described a pigeon liver extract system which readily converted acetate to long chain fatty acids if supplemented with Mg^{++}, DPN^+, ATP and CoA. Popjak and Tietz (15) found ATP, CoA and DPN^+ were needed for acetate incorporation by rat mammary gland tissue. Hele et al (16) in rabbit mammary glands found ATP, CoA, Mg^{++}, cysteine and DPNH were required. In order for avocado particles to incorporate acetate into long chain fatty acids, Stumpf and Barber (17) found a requirement for ATP, CoA and Mn^{++}.

It was believed until recently that lipid synthesis was largely a reversion of β-oxidation. Stansley and Beinert (18), using a highly purified β-oxidation enzyme system and acetyl CoA as substrate, found little butyryl CoA and no longer chained acids. They blamed this on the poor Keq of the thiolase reaction of the reverse β-oxidation chain.

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* TPN^+ and TPNH--triphosphopyridine nucleotide oxidized and reduced form
ATP--adenosine triphosphate
DPN^+ and DPNH--diphosphopyridine nucleotide oxidized and reduced form.

To explain the lack of success by earlier workers in reversing β-oxidation, Langdon (19) suggested that TPN^+-ethylene reductase (an enzyme he discovered) reduced double bond systems to saturated acyl CoA derivatives in place of the reverse thiolase reactions. Seubert, Greull and Lynen (20) added highly purified β-oxidation chain enzymes to TPN^+-ethylene reductase of pig liver mitochondria and noted production of some fatty acid from hex-2-enoyl CoA and acetyl CoA-C^{14}.

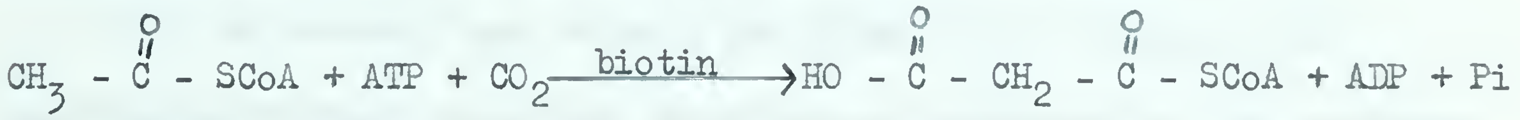
Wakil (21) in 1957 prepared highly purified fractions of pigeon liver. Of the several fractions he separated, none by themselves were active, but some combinations synthesized long chain fatty acids from acetate. In further studies, Porter et al (22) noted that ATP, CoA, DPNH, TPN^+, Mn^{++}, isoctrate and a sulfhydryl compound were needed as co-factors to obtain highest activity. In this system no short chain acids (possible intermediates) were found to accumulate. Using chicken liver Tietz (23) found similar co-factor requirements.

In 1954 Popjak and Tietz (15) noted that malonate added to fractions of lactating rat mammary gland markedly increased the rate of fat synthesis. Later malonyl CoA was identified as an intermediate in the conversion of acetyl CoA to fatty acids in avian liver extracts (24, 25).

Ganguly (26) reported that 2-C^{14}-malonyl CoA is converted to higher fatty acids by a variety of animal tissues. He found that malonyl CoA was always a more efficient precursor than acetyl CoA. Adding a carboxylase to bovine liver extract increased the conversion of acetyl CoA to fatty acids seven-fold, thus showing that in this system the carboxylation of acetyl CoA to malonyl CoA was limiting.

Klein (27) reported that using yeast homogenates, in absence of CO_2, acetate was diverted to a non-saponifiable fraction but in the presence of carbon dioxide a five-fold increase in fat synthesis occurred. Brady and Gurin (28) earlier reported that conversion of octanoate to long chain fatty acids occurred readily in bicarbonate buffer but not in phosphate buffer. Lyon et al (29) reported similar results with rat tissue, as did Squires et al (13) with avocado particles, while Gibson et al (30, 31) noted that $C^{14}O_2$ was not incorporated but apparently played a catalytic role.

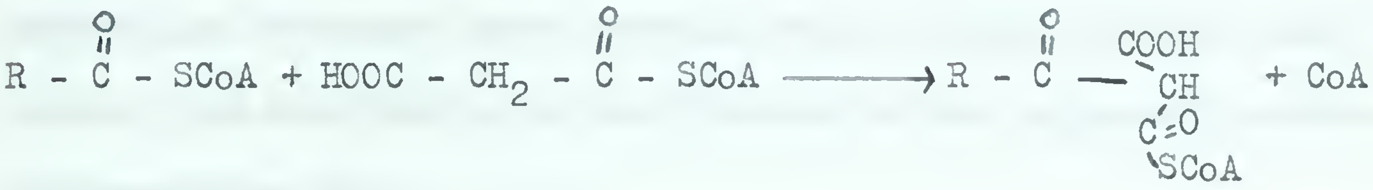
Kurtz and Miramon (32) in 1957 culturing flax embryos found that for optimum fat synthesis from acetate-1-C^{14}, 1 to 100 μgm of biotin were required depending on the age of the tissue. The biotin played a role in carboxylation of acetyl CoA to malonyl CoA. Lynen et al (33) studied a similar reaction--the carboxylation of β-methyl crotonyl CoA to β-methyl glutaconyl CoA by <u>Mycobacterium</u> extract. It was shown that the specific activity of the carboxylase was proportional to the amount of biotin bound by the enzyme protein. From these observations, it was concluded that the reaction taking place was a carboxylation of the acetate molecule to form malonyl.



$$\text{CH}_3 - \overset{\text{O}}{\overset{\|}{\text{C}}} - \text{SCoA} + \text{ATP} + \text{CO}_2 \xrightarrow{\text{biotin}} \text{HO} - \overset{\text{O}}{\overset{\|}{\text{C}}} - \text{CH}_2 - \overset{\text{O}}{\overset{\|}{\text{C}}} - \text{SCoA} + \text{ADP} + \text{Pi}$$

Wakil and Wakil and Ganguly (34, 35), studying conversion of acetyl CoA to malonyl CoA and then to higher fatty acids, found that $TPNH_2$ was needed. It was also reported that substituting butyryl CoA or octanoyl

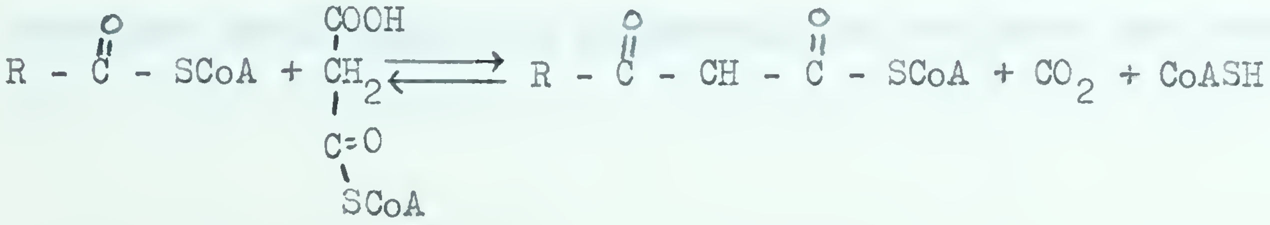
CoA for acetyl CoA resulted in their incorporation into higher fatty acid. Studies with isotopic tracers showed that only one-eighth of the carbon atoms of palmitate had their origin in acetate, whereas the rest originated in malonate. Thus it is suggested that elongation of the fatty acid carbon chain is via condensation of one molecule of a saturated acyl CoA with one molecule of malonyl CoA.



$$R - \overset{\overset{O}{\|}}{C} - SCoA + HOOC - CH_2 - \overset{\overset{O}{\|}}{C} - SCoA \longrightarrow R - \overset{\overset{O}{\|}}{C} - \underset{\underset{SCoA}{C=O}}{\overset{COOH}{CH}} + CoA$$

Therefore with acetyl CoA the two terminal carbon atoms of the fatty acid are from acetyl CoA while the rest of the carbons are from malonyl CoA. With butyryl CoA the four terminal carbon atoms would be derived from butyryl CoA. Horning et al (36) noted that TPNH was needed for fat synthesis. They observed that substituting various branched chain saturated acyl CoA derivatives for acetyl CoA resulted in the formation of the higher branched chain fatty acids expected. To support the above equation Steberl et al (37) found fractions of avian liver extracts incubated with malonyl CoA and a short acyl CoA, accumulated the expected chromatographically identified product (β-ketodicarboxylic acid).

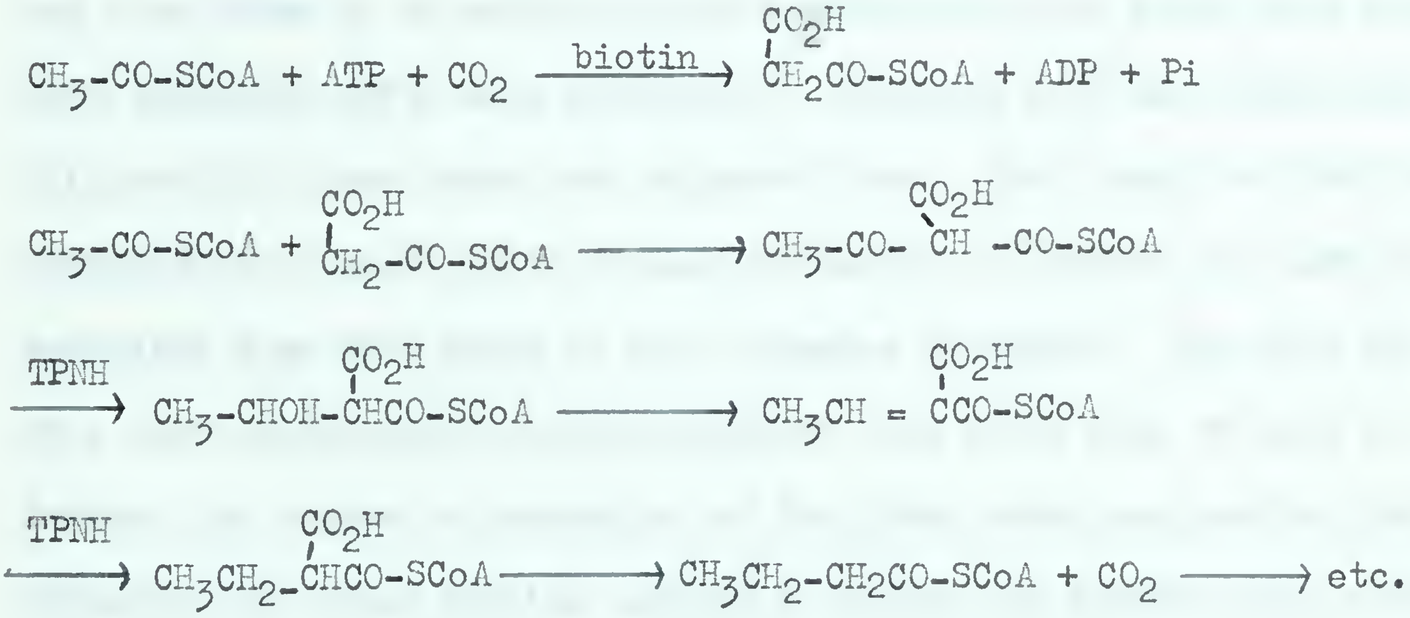
It is noteworthy that Vagelos and Alberts (38) working with Clostridium kluyveri described an alternate mechanism for the condensation of acyl CoA with malonyl CoA. They found it to be a condensation reaction of malonyl CoA and acyl CoA with carbon dioxide being evolved.



$$R - \overset{\overset{O}{\|}}{C} - SCoA + \underset{\underset{SCoA}{C=O}}{\overset{COOH}{CH_2}} \rightleftarrows R - \overset{\overset{O}{\|}}{C} - CH - \overset{\overset{O}{\|}}{C} - SCoA + CO_2 + CoASH$$

It seems, therefore, that the identity of the initial product of condensation of malonyl CoA with saturated acyl CoA derivatives is not known with certainty. Also any further intermediates are in doubt as no intermediate length compounds have been found to accumulate during synthesis of long chain fatty acids (1, 3, 4, 35). It has been found that CoA esters of β-keto, β-hydroxy or α,β-unsaturated acids are not metabolized by avian liver extracts which synthesize fatty acids from malonyl CoA thus failing to confirm some of the suggested reactions and intermediates (39).

Wakil, taking into account the above observations and realizing the importance of malonyl CoA, has proposed the following scheme for fatty acid synthesis.



$$CH_3\text{-CO-SCoA} + ATP + CO_2 \xrightarrow{\text{biotin}} \overset{CO_2H}{CH_2}\text{CO-SCoA} + ADP + Pi$$

$$CH_3\text{-CO-SCoA} + \overset{CO_2H}{CH_2}\text{-CO-SCoA} \longrightarrow CH_3\text{-CO-}\overset{CO_2H}{CH}\text{-CO-SCoA}$$

$$\xrightarrow{TPNH} CH_3\text{-CHOH-}\overset{CO_2H}{C}\text{HCO-SCoA} \longrightarrow CH_3CH = \overset{CO_2H}{C}\text{CO-SCoA}$$

$$\xrightarrow{TPNH} CH_3CH_2\text{-}\overset{CO_2H}{C}\text{HCO-SCoA} \longrightarrow CH_3CH_2\text{-}CH_2\text{CO-SCoA} + CO_2 \longrightarrow \text{etc.}$$

Earlier Rittenberg and Bloch (40) found by measuring the incorporation of $D_3C\text{-}C^{13}OOH$ into liver and carcass fatty acids of intact rodents, that one deuterium atom accompanied each atom of C^{13} incorporated into saturated fatty acids. On degradation it was found that deuterium was

evenly distributed on the carbon chain of oleic which was isolated. These results support approximately the scheme proposed by Wakil.

In a recent study by Wakil et al (41) on fatty acid synthesis from acetyl CoA by rat liver mitochondria extracts it is reported that ATP, TPNH, DPNH and a heat-stable dialyzable co-factor are essential for activity; neither bicarbonate nor malonyl CoA was required. It seems that this enzyme system may be similar to that described by Seubert et al (20) as mentioned previously with reverse β-oxidation enzymes and TPN^+ reductase in the mixture.

Hilditch (42) reviewed some aspects of biosynthesis of unsaturated eighteen carbon fatty acids. Several workers found an increase in the degree of unsaturation during maturation (43, 44, 45, 46). This was at one time taken to be evidence that unsaturated fatty acids were formed from saturated fatty acid precursors. Hilditch (42) and Barker and Hilditch (47) from their work disputed this. They found the fatty acid composition of seeds grown in warm localities of Africa was much more saturated than when grown in cool climates of Africa. But when the seeds from these different African localities were grown side by side in England the degree of saturation of the fatty acids was similar (48). Grindley (49) found similar results in winter and summer grown sunflower in Khartaum. Recent studies by McGregor and Carson (50) with linseed grown at Morden, Manitoba in the south and Fort Vermillion, Alberta in the north support the above results. Hilditch believes these observations to mean that more saturated acids are formed from more unsaturated ones. This he concludes on the assumption that in the cooler climate the

synthesis does not go to completion whereas in a warm climate it does. The incomplete fat then would be quite unsaturated, whereas the complete fat is much more saturated.

At different temperatures the composition of saturated fatty acids does not vary but the composition of the unsaturated acids does (42). For this reason Hilditch (51) suggests the two are formed by different pathways. He proposes saturated acids are formed by acetyl condensations while unsaturated acids by pyruvic condensations.

Invitro work by Squires and Stumpf (11), Porter and Long (52) and by Tietz (23) shows that in enzyme extracts the predominating acid formed is a saturated one, usually palmitic. Unsaturated acids are formed in smaller proportions. Stumpf and James (53), studying the incorporation of radioactive acetate into long chain fatty acids by chloroplast preparations of lettuce leaf homogenates, found 57% of the label in palmitic and 38% in oleic acid. Since these reactions took place in a relatively short time it may indicate that saturated acids are formed first and unsaturated acids are formed from them or unsaturated acids are formed by a different, slower pathway.

Several studies have dealt with changes in fatty acid composition in oil plants during different stages of development. Crombie (1), working with the maturing kernel of oil palm, noted that the fat in the mature kernel contained a mixture of nine fatty acids including some oleic and linoleic. At early stages of development the unsaturated acids formed a much larger fraction of the total than they did at later stages. However, in the maturing exocarp of the oil palm Crombie and Hardman (2) found very few changes in the fatty acid composition during development.

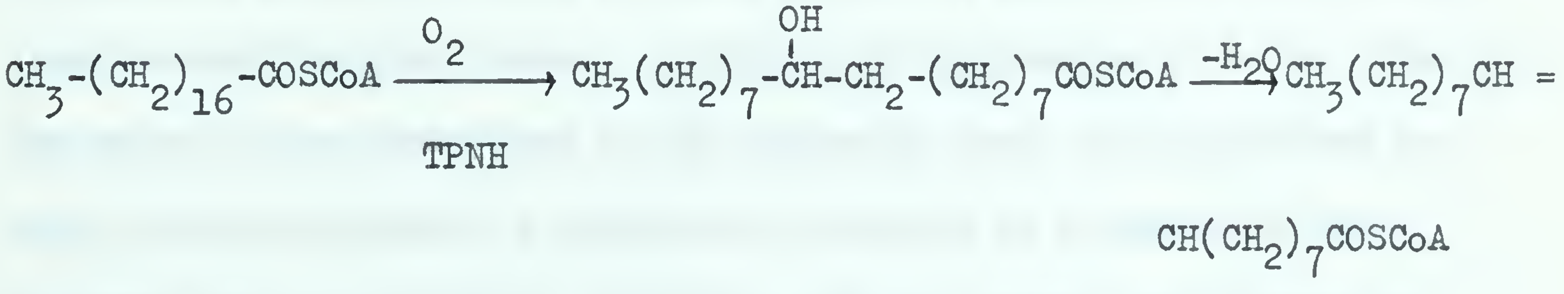
Painter (44) collected flax bolls at different times during development. Total fatty acids increased in amount during development and the iodine number increased significantly. Painter interpreted these results to mean that unsaturated fatty acids are formed by desaturation, but drew attention to the fact that unsaturated acids may be formed later than saturated ones, or more slowly. Also studying linseed oil composition during development, Hashad et al (54) found that the iodine number increased during the entire development period but at a slower rate near maturity. Smith and Kurtz (55) found in *in vivo* and *invitro* studies with developing flax embryos that the saturated acids palmitic and stearic and the monounsaturated acid oleic develop first while linoleic and linolenic arise later.

In 1954 Simmons and Quackenbush (56) harvested soybeans at successive stages of maturity and determined amounts of saturated, oleic, linoleic and linolenic acids present. They found a continuous increase in amounts of each acid present. The iodine number and per cent linolenic acid decreased some during the early stages. The linoleic acid percentage and per cent oil increased steadily till the fiftieth day, remaining constant after this. Oleic and saturated acid percentage fluctuated. No evidence of dehydrogenation of saturated fatty acid was obtained either in oil analysis or analysis of tissue for dehydrogenose activity.

Hopkins and Chisholm have studied oil development in seed of *Helianthus annus* L. (45) and in *Asclepias syriaca* L. (57). In *Helianthus annus* L. development no evidence of any intermediate accumulating could

be found. The amount of each acid present increased to maturity. Oleic acid accumulated most rapidly at first but at the mid-point it was overtaken by linoleic. It was mentioned that possibly oleic acid was converted to linoleic. Because there is little change in amounts of saturated long chain acids, it is thought they are not involved in the synthesis of unsaturated fatty acids. In Asclepias syriaca L. there was a sudden change in the fatty acid composition at a middle stage of development when the percentage of hexadecenoic and octadecenoic acids fell and that of linoleic rose. By weight per 1000 seeds linoleic acid increased steadily, while octadecenoic and hexadecenoic increased slowly. The proportions of the other acids remained more or less constant for an eleven week period after blooming.

Bloomfield and Bloch (58) in 1960 reported on studies of desaturation of palmitic to palmitoleic acid and of stearic to oleic by yeast extracts. They showed that the fatty acids participate in this reaction as the CoA thioesters. To convert palmityl CoA to palmitoleyl CoA the particulate yeast fraction needs only TPNH and oxygen. It was postulated that desaturation involves formation and dehydration of a hydroxyl intermediate.



$$CH_3\text{-}(CH_2)_{16}\text{-}COSCoA \xrightarrow[TPNH]{O_2} CH_3(CH_2)_7\text{-}\overset{OH}{CH}\text{-}CH_2\text{-}(CH_2)_7COSCoA \xrightarrow{-H_2O} CH_3(CH_2)_7CH = CH(CH_2)_7COSCoA$$

Strong support for this was found by Lennarz and Bloch (59) using tritium labelled 9-hydroxy stearic acid which was converted to oleic acid by crude yeast extracts.

In 1954 Simmons and Quackenbush (60) reported work using excised soybean stems dipped in isotopic sucrose, collecting pods at various intervals and analyzing the oil for composition and label. It was found that oleic acid was most highly labelled and that the specific activity increased with time. Linoleic and linolenic acids were less labelled but during a period when oleic acid specific activity increased 2-fold, linoleic activity increased 4-fold and linolenic 7-fold. During the period of increase in activity the relative fatty acid composition of the oil remained constant, strongly suggesting conversion of oleic to other fatty acids.

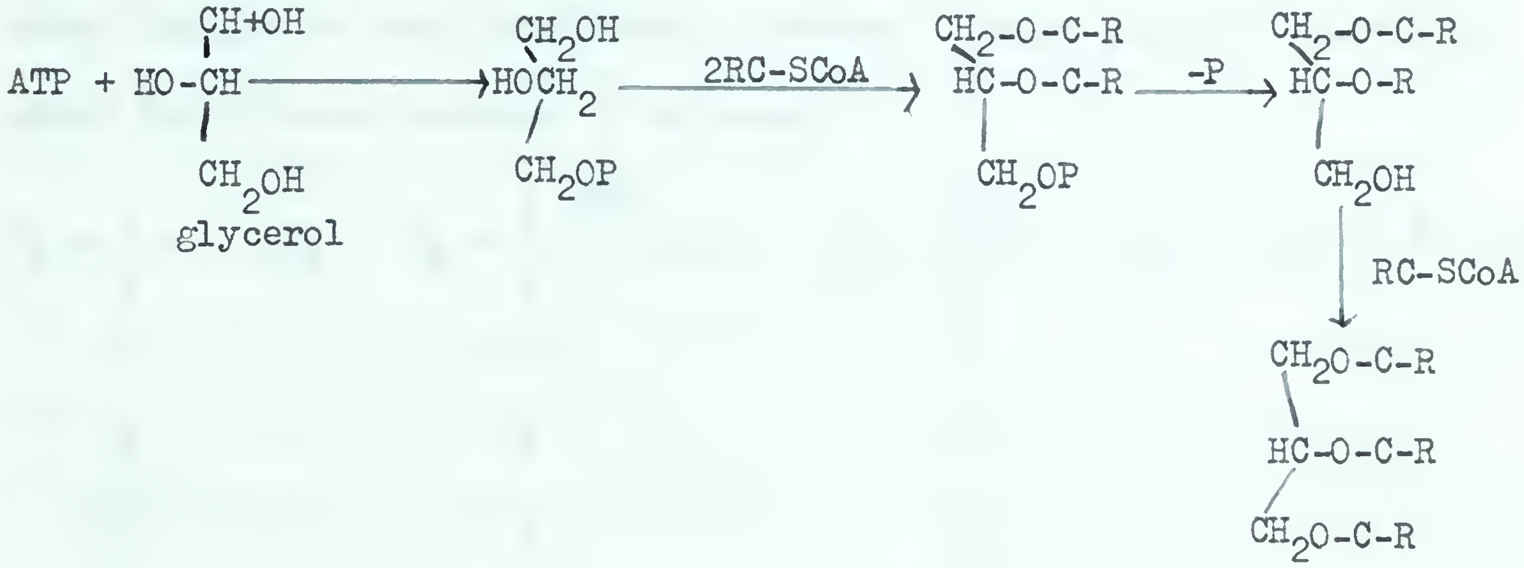
Ching Yaun and Bloch (61) supplied labelled oleic acid to yeast cells. Essentially all the label found in the fatty acids after incubation was either in oleic acid or in multiple unsaturated fatty acids. They propose that desaturation to acids of equal chain length is the principle metabolic pathway of oleic acid in yeast. They could not show that the tritium labelled hydroxy acid ricinoleate, a possible intermediate, was used to form linoleate.

James (62) found that isolated castor-oil plant leaves readily take up labelled acetate to form labelled myristic, palmitic, stearic, oleic, linoleic and linolenic acids. Studying the percentage of total label in the acids it was found that at the beginning oleic acid contained the most activity but that it decreased later with an increase in the activity of linoleic acid. Palmitic acid and stearic acid activity remained relatively constant in this period. The evidence suggests that neither palmitic nor stearic acids are precursors of oleic acid but that the latter is converted to linoleic acid. This was supported by the

results of a leaf-feeding experiment. The activity of oleic acid was recovered mainly in linoleic acid. Linolenic acid, although the major component of leaf fat, was synthesized much more slowly, labelling occurring only after 24 hours and the increase being parallel to a fall in linoleic activity, suggesting the latter is its precursor.

When stearic-C^{14} acid was fed to rats the lipid of the liver contained 57% stearic-C^{14} and 27.5% oleic-C^{14}. This suggests dehydrogenation of stearic to oleic acid (63).

Paulus and Kennedy (64) have proposed the following scheme for the synthesis of triglycerides on the basis of studies by many workers:



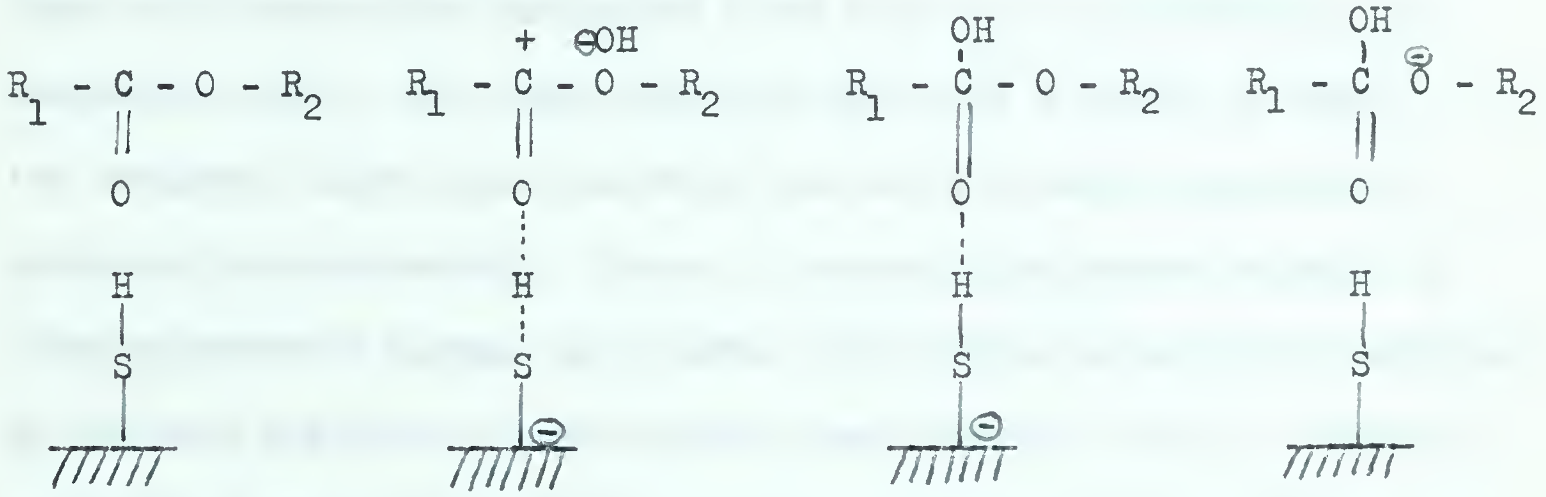
The scheme requires ATP, fatty acids and glycerol.

Crombie and others (1, 2) found a 100-fold increase in triglyceride content of oil palm during a period 10 to 20 weeks after pollination. Hopkins and Chisholm (45, 57) obtained similar results in Helianthus annus L. (sunflower) and in Asclepias syriaca L., and Painter (44) found rapid oil deposition in developing flax.

Fatty acid and lipid breakdown

In plants and in animals at one stage or another there is breakdown of lipid material. The first step involves splitting acids off the triglyceride molecule. This is accomplished by lipase, a sulfhydryl enzyme.

Scott (65) put forward a mechanism for pancreatic lipase activity. The theory postulates that the negatively charged carbonyl oxygen of the triglyceride ester combines with an electrophilic group of the enzyme, thought to be the -SH group. The electron density at the carbonyl makes the carbonyl carbon positive in charge and a hydroxyl group linkage is then facilitated. Further linkage with the sulfhydryl group facilitates cleavage of the acid.



Singer and Hofstee (66) were able to show wheat germ lipolytic activity on a large series of simple esters and on mono and tri-glycerides. The lipase was inhibited by sulfhydryl reagent but evidence was obtained that the sulfhydryl groups of this lipase were not directly involved in its activity. Singer (67) suggested that the sulfhydryl groups might be located so close to the center of activity that the formation of complexes of the

inhibitors with the sulfhydryl groups might interfere with the molecules of substrate approaching. Gawron and coworkers (68) decided from their work with wheat germ lipase that the sulfhydryl group of the enzyme did not participate in hydrolysis.

The role of lipase during germination of several oil seeds was studies invivo by various workers. Lipase activity of rapeseed was reported to increase after germination (69). It was found by Johnson and Sell (70) that the oil of tung seed is utilized during germination and that this utilization is accompanied by the formation of free fatty acids. This suggests that the glycerides of tung oil are hydrolized before being broken down further.

In 1956 Crombie and Comber (71) found only slight changes in the fatty acid composition during the first four days of germination of watermelon seeds. But after four days there was a period of rapid fat breakdown (high lipase activity) and all the acids except oleic decreased proportionately. Oleic, it seemed, disappeared faster. In other experiments Hardman and Crombie (72) germinated sunflower seedlings in the dark and found essentially the same results; that is, the lipid composition remained constant throughout germination. Free fatty acids were found not to accumulate.

White (73) reported that germinating cottonseeds both in the light and in darkness utilized the fatty acids in proportion to the amount originally present in the seed. During all stages there was very little free fatty acid present.

Kartha and Sethi (74) on the basis of work with germinating peanut seeds report that the use of reserve fats in germinating seeds takes place in a non-selective manner, and any changes which take place do so only after the fatty acids have been hydrolized from the glyceride fraction. On the other hand, Rabari et al (75) also working with germinating peanuts report that saturated fatty acids are metabolized at a greater rate than unsaturated fatty acids during germination.

Discussing their work, Kartha and Sethi (76) stated that the plant can exercise great control over lipase activity and can restrict the lipolytic hydrolysis of fat during germination to sufficiently low rates that the liberated acids are immediately used up without allowing the acids themselves or lower acids produced by them by β-oxidation to accumulate to any extent. Because free fatty acids are not present in measurable quantities, their presence may not be desirable to the plant.

Bradbeer (11) noted that there was little accumulation of free fatty acid during the germination of etiolated sunflower seedlings. However, Gamborg and Zalik (77) report a small accumulation of free fatty acids during the germination of sunflower seedlings.

Knoop (78) described β-oxidation of fatty acids in 1904. He tagged fatty acids by means of a phenol group (not easily degraded) and fed them to animals. From products formed as elimination products, Knoop decided that oxidation of fatty acids occurred in such a way that there was a loss of two carbon atoms due to oxidation at the β-carbon atom.

Shoenheimer and Rittenberg (79) confirmed the general concept of β-oxidation by isolating labelled palmitic acid after having fed deutero-stearic acid.

Grace in 1939 (80), working with plants, found an alternation of activity in ω-substituted aryl carboxylic acid, in growth promoting properties. Only those acids with an odd number of methylene groups in the side chain had the activity of 1-naphthyl-acetic acid, which has one methylene group. It was suggested by Synerholm and Zimmerman (81) that β-oxidation would explain this.

Results to support the concept of β-oxidation in plants were obtained by Fawcett et al in 1954 (82). They supplied members of a homologous series of ω-phenoxy acids to flax seedlings and after a suitable time period they assayed the tissue for phenol. Sizable amounts of phenol were produced only by units with an even number of side chain methylene groups.

The first enzyme in the β-oxidation chain, acetic thiokinase, was first found in spinach, wheat and other plant tissues by Millerd and Bonner (83). It catalized the conversion of acetate, coenzyme-A and ATP to acetyl CoA.

The β-oxidation chain has been worked out in much detail in animals. The result of the work is summarized in the following scheme (84):

1. $R\text{-}CH_2\text{-}CH_2\text{-}COO^- + ATP + CoASH \xrightleftharpoons{Mg^{++}} R\text{-}CH_2\text{-}CH_2\text{-}COSCoA + AMP + PPi^-$

enzymes: a. acetic thiokinase (acetic and propionic acid)
b. fatty acid thiokinase (4C to 12C fatty acids)
c. long chain fatty acid thiokinase

2. $R-CH_2-CH_2-CO-SCoA+\text{flavoprotein enzyme} \rightleftharpoons R-CH=CH-CO-S-CoA+\text{reduced flavoprotein}$

   enzymes: a. G (green flavoprotein) 4C to 8C acids

   b. Y (yellow flavoprotein) 8C to 12C acids

   c. Y' (yellow flavoprotein) 8C to 18C acids

3. $R-CH=CH-CO-S-CoA+H_2O \rightleftharpoons R-CHOH-CH_2-CO-S-CoA$

   enzyme: enol hydrase (4C to 18C acids)

4. $R-CHOH-CH_2-CO-S-CoA+DPN^+ \rightleftharpoons R-CO-CH_2-CO-S-CoA+DPNH+H^+$

   enzyme: β-hydroxyacyl dehydrogenase (specific for DPN)

5. $R-CO-CH_2-CO-S-CoA+CoASH \rightleftharpoons R-CO-SCoA+CH_3-CO-S-CoA$

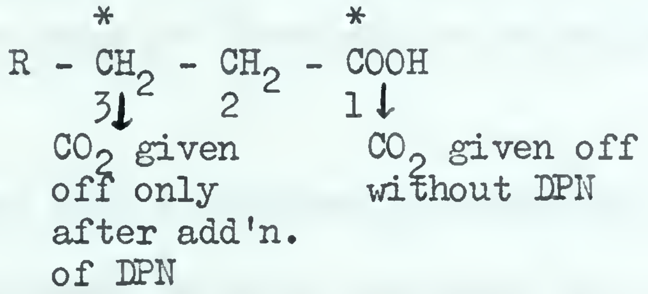
   enzyme: β-ketoacyl thiolase

The acyl Coenzyme A derivative can then undergo further β-oxidation beginning with step 2.

Working with cell-free plant systems, Stumpf and Barber (85) were able to demonstrate β-oxidative degradation of a large number of fatty acids. To do this the following requirements had to be met: (1) fresh peanut mitochondria (2) low fatty acid and (3) many cofactors (ATP, CoA, DPN, TPN, Mn^{++}, a TCA acid and glutathione). In animals CoA, TPN and DPN were not needed (86, 87). A large number of cofactors were also found necessary to oxidize Krebs cycle acids (88).

The acetyl CoA formed by β-oxidation can be utilized by the plant in several ways. As previously mentioned, it is a compound of central importance in metabolism.

α-oxidation was first reported in 1952 by Newcombe and Stumpf (89). They found that cell free fractions of germinating peanut seedlings released $C^{14}O_2$ from palmitate-1-C^{14} with no cofactors, but DPN^+ was needed to release $C^{14}O_2$ from palmitate-3-C^{14}.



$$\overset{*}{\underset{3}{CH_2}} - \underset{2}{CH_2} - \overset{*}{\underset{1}{COOH}}$$

Humphreys et al in 1954 (90) found that the thiol ester palmityl-1-C^{14}CoA was an ineffective substrate for CO_2 release by peanut cotyledon extract. They also found that labelled internal carbons of palmitate released labelled CO_2 if DPN^+ was present but that the release decreased with distance from the acid carbon. Humphreys and Stumpf (91) obtained a soluble enzyme system from the peanut cotyledon fraction which gave off CO_2 from fatty acids with cofactor requirements similar to the above.

Through the work of Castelfranco et al (92) and Stumpf (93) it was found that hydrogen peroxide (H_2O_2) was needed to peroxidize the saturated fatty acids. At least two enzymes are required in the internal oxidation of long chain fatty acids (94). The first is fatty acid peroxidase which peroxidatively decarboxylates the acid to an aldehyde and CO_2:

$$R - CH_2 - CH_2 - COOH \xrightarrow[\text{fatty acid peroxidase}]{H_2O_2 \quad \nearrow CO_2} RCH_2CHO$$

and the second is a DPN^+ specific aldehyde dehydrogenase oxidizing the aldehyde to an acid:

$$R - CH_2 - CHO \xrightarrow[\text{aldehyde dehydrogenase}]{DPN^{+} \quad DPNH} RCH_2COOH.$$

The acid can undergo further α-oxidation. The activity of the peroxidase seems limited to the 14 carbon to 18 carbon acids with the 15 carbon acid being the most active substrate.

Other types of α-oxidation mechanisms have also been studied (95, 96).

Stumpf (97) by following isotopically labelled glycerol in germinated peanut cotyledon extracts concluded that glycerol was metabolized in plants by being converted to triose phosphate. This was then respired or converted to sugar.

$$\begin{tikzcd}
\text{glycerol} \arrow[r, "ATP"] & \alpha\text{-glycerol-phosphate} \arrow[r, "-2H", "\text{cytochrome C}"'] & \text{triose-phosphate} \arrow[r] \arrow[dr] & \text{sugar, etc.} & \\
 & & & \text{Krebs cycle} \arrow[r] & CO_2
\end{tikzcd}$$

The basic observations of a decrease in the fat content during germination accompanied by a carbohydrate increase and by low R.Q. values have been described many times. For example, an ungerminated castor bean contains about 260 mgm. of fat and 15 mgm. of carbohydrates. After eight days of germination the total fat content in the seedling falls to 50 mgm. and the carbohydrate rises to 230 mgm., this despite the fact carbohydrate is being used for respiration (98). Because the respiratory quotient of endosperm tissue during this stage is 0.35, while in the growing seedling it is 1.0, it seems obvious that the interconversion is strictly confined to endosperm tissue (99).

Beevers (100) showed that during the interconversion stage glycerol is quite efficiently converted to sugar but he states this can account for very little of the carbohydrate formed. Thus acetyl-CoA formed by β-oxidation must be responsible for the major portion of carbohydrate formed. Indeed Beevers (101) established that slices of endosperm tissue readily incorporate acetate into carbohydrate.

The glyoxylate shunt of the Krebs cycle elucidated by Kornberg and Krebs (5) seems to be the obvious means of the interconversion. The shunt is illustrated on the diagram on page 22.

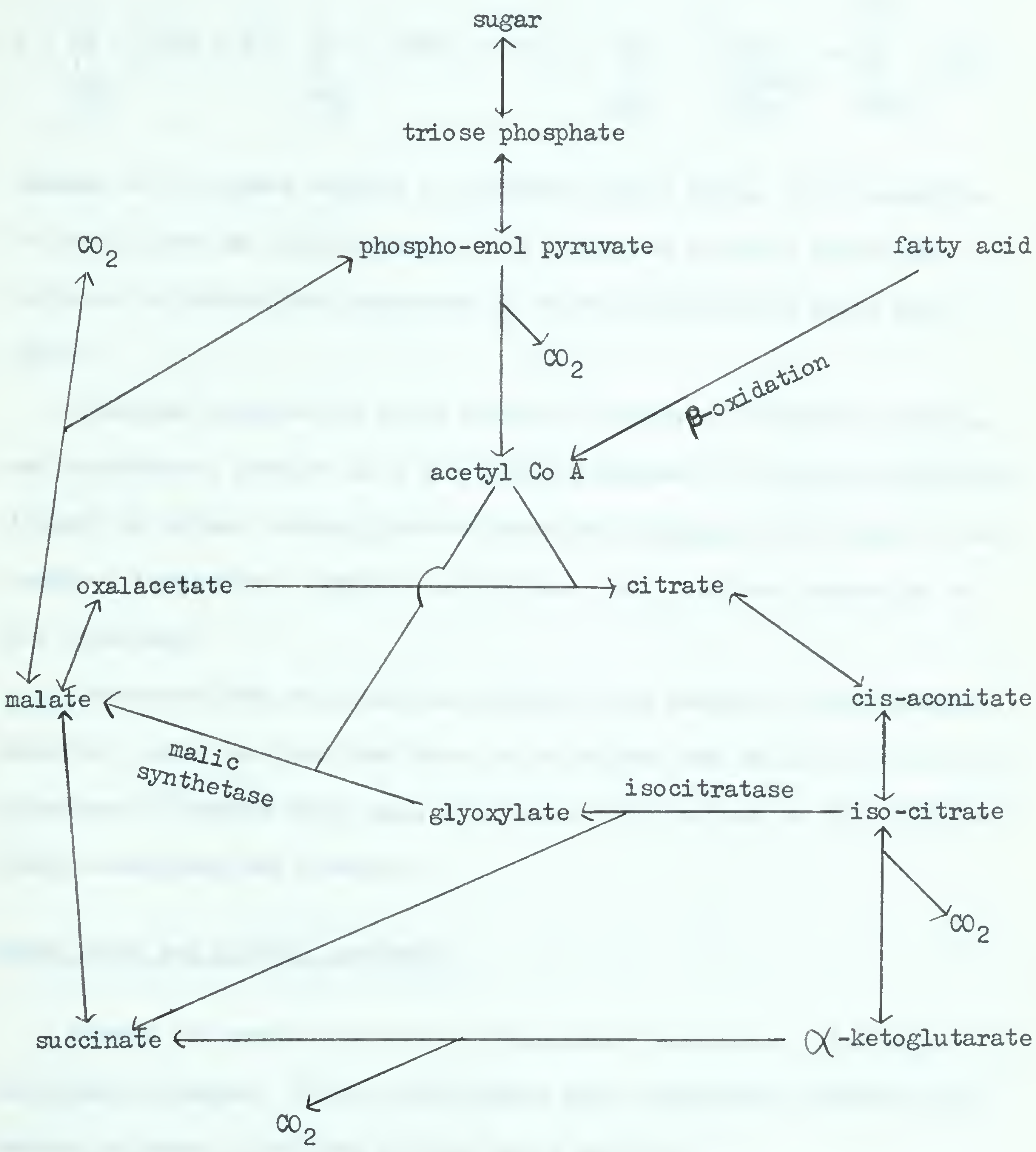
Isocitratase, one of the two enzymes exclusive to the glyoxylate shunt, was found by Carpenter and Beevers (102) to be confined to tissues rich in fat, especially in germinating seedlings. It was found only in those tissues actively converting fat to carbohydrate. The peak activity of the enzyme was found to be right at the stage of most rapid fat breakdown. Malate synthetase, the other enzyme, was found to be similar in activity to isocitratase (103).

II. Protein Metabolism

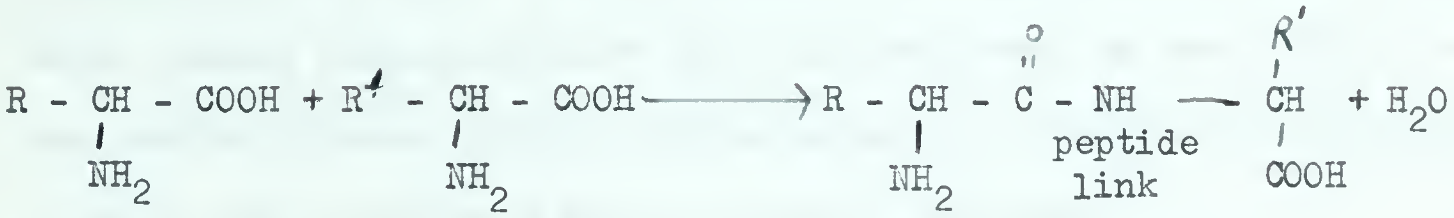
Proteins represent organic substances of large molecular size. Each molecule of protein is composed of very many atoms. For this reason the protein chemist has centered his attention on a variety of relatively small molecules, amino acids, of which protein material is composed. An amino acid is a hydrocarbon acid with an amino group at the α-carbon. Proteins of different origin and of different types have different amino acid

Diagram. Glyoxylate cycle.

Diagram


sugar
triose phosphate
phospho-enol pyruvate
CO_2
CO_2
fatty acid
β-oxidation
acetyl Co A
oxalacetate
citrate
malate
cis-aconitate
malic synthetase
isocitratase
glyoxylate
iso-citrate
CO_2
succinate
α-ketoglutarate
CO_2

complements. The basic linkage of amino acids in protein is the peptide linkage



$$R - \underset{\underset{\textstyle NH_2}{|}}{CH} - COOH + R' - \underset{\underset{\textstyle NH_2}{|}}{CH} - COOH \longrightarrow R - \underset{\underset{\textstyle NH_2}{|}}{CH} - \overset{\overset{\textstyle O}{\|}}{C} - \underset{\text{peptide link}}{NH} - \overset{\overset{\textstyle R'}{|}}{\underset{\underset{\textstyle COOH}{|}}{CH}} + H_2O$$

Because of the great variety of different amino acids, it is possible to have almost an infinite variety of different proteins since one molecule of protein may represent up to one half million amino acid units.

Proteins compose the major portion of enzymes. Thus the position and function of protein in a biological organism is of great importance. A plant or animal lacking protein lacks the machinery with which it can respire, synthesize, degrade, assimilate, etc., products essential to its existence.

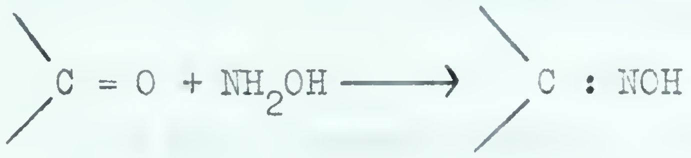
Because of the very complex structure and makeup of proteinaceous material, much work has been done on its nature and on its role in life processes. Despite this, much work must still be done in this field to fully understand the details.

Amino acid and protein synthesis

Plants can synthesize their nitrogeneous components using only inorganic nitrogen. Knoop, fifty years ago, postulated reductive formation of amino acids from α-keto acids and NH_3.

The nitrogen required for amino acid synthesis is incorporated into the carbon skeleton as NH_3 or perhaps as NH_2OH (hydroxylamine). Hydroxy-

lamine is the last intermediate in the reduction of NO_3^- (the form of nitrogen in the soil) to NH_3. A specific enzyme, hydroxylamine reductase, which reduces hydroxylamine to NH_3, has been found by many authors as reviewed by Virtanen (104). Hydroxylamine is a very reactive compound, forming oximes readily with many carbonyl compounds.



Thus if hydroxylamine is not immediately reduced to ammonia it is fixed in the organisms as the oximes of acids such as glyoxylic, pyruvic, oxaloacetic and α-ketoglutaric. So the accumulation of free hydroxylamine is not probable. The fact that oximes are formed from glutamic and aspartic acids as well as from glutamine and asparagine has been demonstrated in many organisms (105, 106, 107).

If these oximes were reduced in the organisms, the corresponding amino acids could be formed from them. This reduction, however, has not as yet been substantiated. Maurer (108) found that alanine was formed from oximinopyruvic acid with an actively fermenting brewer's yeast, but many unphysiological compounds are reduced by fermenting yeast.

In aerated *Torulopsis* (yeast) suspensions there is a rapid decrease of oximes (104), making it probable that some reaction occurs by which the oximes are decomposed.

When ammonium nitrogen served as the only source of nitrogen for *Torulopsis*, no bound hydroxylamine was formed (109).

On the basis of these and other observations, Virtanen suggested the following pathway of nitrogen incorporation into amino acids (104):

$$\begin{tikzcd}
NO_3^- \arrow[r] & NH_2OH \arrow[rr] \arrow[d, shift left] & & NH_4^+ \arrow[d, shift left] \\
& \text{oximes} \arrow[u, shift left] \arrow[rr, dashed, "\text{(not verified)}"'] & & \text{amino acids} \arrow[u, shift left]
\end{tikzcd}$$

The first enzyme synthesis of an amino acid was not a common amination of a keto acid. In 1926 Quastel and Woolf (110) noted the synthesis of L-aspartic acid by E. coli from fumarate and NH_4Cl.

$$\text{fumarate} + NH_4Cl \longrightarrow \text{L-aspartic acid}$$

Aspartase, the enzyme catalizing the above reaction, was found in many, but not all, microorganisms and in some higher plants (111).

V. Euler et al in 1938 reported (112) the synthesis of L-glutamic acid by the enzyme glutamic acid dehydrogenase which they isolated.

$$\text{L-glutamic acid} \rightleftarrows \alpha\text{-ketoglutaric} + NH_3$$

This enzyme was found in many different types of organisms and work has shown it to be of great importance for the synthesis of L-glutamic acid.

The amino acids which are formed directly by combination with ammonia are referred to as 'primary' amino acids, and those formed from precursors already having an amino group are referred to as 'secondary' amino acids.

At present there are three known primary amino acids: L-aspartic and L-glutamic as noted above and alanine formed by alanine dehydrogenase isolated by Goldman (113).

$$\text{L-alanine} + DPN^+ \rightleftharpoons \text{pyruvate} + NH_4^+ + DPNH$$

There are possibly several other primary amino acids (114, 115).

In those organisms in which the amino acid synthesis has been studied in detail it seems that alanine is a secondary amino acid. Burris, Wilson and coworkers indicate that NH_3 is the key compound of nitrogen incorporation and that L-glutamic acid is the only primary amino acid (116). But in many experiments the labelling of glutamic and aspartic has been so nearly equal that aspartic may also have resulted from primary synthesis. Allison and Burris (117) using very short experiment times have recently conclusively proven that in N-fixing microorganisms, aspartic acid is of secondary origin.

Folkes (118) found that at least 75 to 85% of the primary amino acid formation was by way of glutamic acid and its amide glutamine.

The main pathway for the formation of secondary amino acids, transamination, was discovered first in animals by Braunshtein and Kriteman (119). Virtanen and Laine later showed that it also functioned in higher plants (120). Meister et al (121) noted that the α-amino nitrogen of glutamine and asparagine was transaminated with the simultaneous splitting of the amide nitrogen to form ammonia. The transaminase reactions are reversible and thus glutamic acid in the cell could be of either primary or secondary origin. There is thus possible a complicated dynamic state of free amino and free keto acids in the cell. However, the specificity of transaminases is not too well established. Fincham and Boulter (122) suggest that some transaminases are common to many acids.

The glyoxylate cycle could be of some importance in supplying the α-keto acids for the transaminase reactions. This could be a means of supplying the plant with skeletons for amino acids without depleting the supply of these acids for respiration (123).

A striking feature of amino acid metabolism is the "families" of related amino acids. A list with the heads given first follows (124):

glutamic family--glutamic acid, proline, arginine
aspartic family--aspartic acid, methionine, threonine, isoleucine, lysine
serine family--serine, glycine
pyruvic family--alanine, valine, leucine
aromatic family--tryptophan, tyrosine, phenylalanine, histidine.

Roberts et al (125) found competition for C^{14} incorporation among families; the heads, if added, inhibiting the incorporation into the family. Cowie and Walton (126) found that the heads of families were labelled first after adding C^{14}-fructose.

Bilinski and McConnell (127) report a similarity in amount of label in members of the glutamic family after injection of acetate into stems of maturing wheat plants.

It is interesting to note the role of glutamine and other amides in amino acid synthesis. Folkes and Yemm (128) found that glutamine was of central importance to the metabolism of barley seedlings. Samparo and Folkes (129) found the free glutamine of the embryo to be relatively low with far more being associated with the peptide fraction. Spragg (130) in a 75% ethanol extract of pea seedlings (supposedly separated out free amino acids) found a conspicuously large amount of glutamine and arginine. In the xylem sap of many plants, Bollard (131) found glutamine and arginine to be generally the most plentiful nitrogen compounds.

Sivaramakishnan and Sarma (132), studying the germination of green Gram seeds, noted a very rapid catabolism of amino acids and presumably a lot of synthesis as well. When labelled glutamic acid was fed, most of the label recovered was CO_2 with some conversion of glutamic to arginine and proline.

During the germination of lettuce seeds, Evanari (133) noted that the concentration of the fourteen amino acids present remained constant during germination.

The synthesis of protein from amino acids seems to go as follows: free amino acids $\longrightarrow$ peptides $\longrightarrow$ polypeptides or protein. Cocking (134) found young barley seedlings, which were fed labelled nitrogen, first had an increase of label in the free amino acids and then in the proteins. Other workers found similar results with other organisms (117, 135). Turba and Essen (136) noted that yeast fed labelled acetate gave curves of incorporation of label into amino acids, peptides and proteins which were parallel and in respective decreasing orders of label intensity.

In the past there has been difficulty in finding a cell-free extract capable of synthesizing protein. However, Webster (137) and others have recently found enzyme preparations capable of accomplishing a net synthesis of protein. Work with these systems should increase our knowledge of protein synthesis.

On the basis of a large number of experiments by many workers, the following steps can now be proposed to occur in the synthesis of many kinds of protein molecules (138):

Step 1--a.a. activation

$$aa + sRNA + ATP \xrightarrow{Mg^{++}} aa - sRNA + AMP + PPi^*$$

Step 2--peptide bond formation

$$n(aa - sRNA) + ribosome \xrightarrow{GTP,\ Mn^{++}} \text{polypeptide attached to ribosome} + n(sRNA)$$

Step 3--release of polypeptide from ribosome

$$\text{polypeptide attached to ribosome} \xrightarrow{ATP,\ Mg^{++}} \text{polypeptide} + \text{ribosome.}$$

There is then further modification of the completed polypeptide chain. Folding, combination by various links and other processes may take place.

The sequential order of the amino acids in the polypeptide seems to be ordered by the RNA located on the surface of the ribosome. It is suggested that the sRNA particle has a particular base order which fits a complementary base order on the RNA of the ribosome. If each sRNA particle is specific for a certain amino acid, then the amino acid order of the protein could be controlled by the RNA of the ribosome (139).

Amino acid and protein breakdown

The breakdown of protein as a means of releasing energy in a plant or other organism is not of as much importance as the breakdown of lipid or carbohydrate. The only time proteinaceous material is utilized for energy is under starvation states.

However, protein is in a constant dynamic equilibrium in a living cell, and thus new protein is constantly being formed while old is being broken down (132, 133).

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* sRNA--soluble Ribonucleic acid.

Enzymes which break down protein material are referred to as proteolytic enzymes. There are two types: (a) proteases, which are nonspecific and attack both protein and peptides, and (b) peptidases, which are specific for peptides breaking them down to their amino acid derivatives.

The breakdown of the amino acids is mainly by reversal of the transaminase reactions and a reversal of the reactions forming primary amino acids. The nitrogen which is removed by amino acid breakdown may be used to form new protein or may be taken up in an amide linkage.

MATERIALS AND METHODS

Materials

The plant material used in this study was No. 1 Registered Redwing flax seed (*Linum usitatissimum* L.) purchased in May, 1961 from Canwest Seed Company, of Edmonton, Alberta.

Studies concerning developing flax seed were carried out by growing plants till blooming and samples of seed bolls were collected at later stages. One group of plants was grown in a field plot in the summer of 1961 and the second in the greenhouse in the spring of 1962. The greenhouse was held at a constant temperature of 21°C. Supplementary lighting was provided by fluorescent lamps from 4 a.m. to 8 p.m. daily.

Plant materials used for germination studies were grown in glass trays (10" x 10" x 6"). The trays were lined with paper towels soaked in Hoagland's No. 1 nutrient solution supplemented with micronutrients (140). Paper towelling was bent over the sides of the tray and dipped into a reservoir of demineralized distilled water. The trays were covered with a glass plate. The germination temperature was held between 20°C and 25°C. One group of plants was given a 16-hour light, 8-hour dark regime; another was kept in the dark.

Total lipids, free fatty acid content and fatty acid composition

Samples of field grown and greenhouse grown seed bolls were collected weekly after blooming. As the oil content increased, fewer bolls were collected per sample.

For germination studies seedlings were collected daily up to 7 days. In all instances duplicate samples were taken for analysis. The samples were freeze-dried, then weighed, ground, and analyzed for total lipid by A.O.C.S. methods (141). The crude material left after extraction was saved for protein analysis.

Aliquots of oil from samples taken after germination in the light were used to determine the percentage of free fatty acids by A.O.C.S. methods (141).

To determine fatty acid composition, triglycerides were methylated using sodium metal as a catalyst suspended in methanol (142). Boron trifluoride was used as catalyst to methylate the free fatty acids (143). The methyl esters were analyzed by gas-liquid chromatography (GLC) (144). An Aerograph Gas Chromatographic Instrument, Model A-100 was used. The column was five foot, $\frac{1}{4}$ inch copper packed with 30% diethylene glycol succinate on crushed fire-brick. The oven temperature was 200°C. Helium flowing through the column at 75 c.c./minute was the carrying gas. A thermal conductivity cell with a 250 milliampere current detected sample components. A high speed recorder coupled with a Disc Integrator recorded the components. Depending on the amount of the most abundant component expected, samples varying from 1 μl to 10 μl were injected.

Total protein, free amino acids and amino acid composition

Total nitrogen was determined in all samples by a Microkjeldahl method (145). Peroxide was added to hasten the digestive process (146). Protein was expressed as nitrogen X6.25.

To prepare samples for free amino acid determinations, five hundred mgm. samples were homogenized for five minutes in water in a Servall omnimixer at 3/4 maximum speed. The homogenate was centrifuged in an angle centrifuge for 15 minutes at 20,000 X g. Five ml. of 5% trichloracetic acid (TCA) was added to the supernatant. It was homogenized for five minutes and centrifuged at 20,000 X g. for 15 minutes. The supernatant was transferred to volumetric flasks, TCA was added to check completeness of precipitation and the solution was made to volume. Five ml. aliquots of this were used for nitrogen determinations. To convert to mgm. free amino acids the factor 6.25 was used.

To determine amino acid composition, the oil-free samples were hydrolyzed by gently refluxing in constant-boiling glass-distilled hydrochloric acid for 24 hours. The hydrolysates were evaporated to dryness on a rotary evaporator and the residue was taken up in 0.2 M citrate buffer (pH 2.2). The amino acid determinations were carried out on a Model 120 Spinco Amino Acid Analyzer.

Radioisotope studies

Sodium acetate-1, 2-C^{14} was administered to developing flax plants in the greenhouse during the stage of rapid fat synthesis (about the second week after blooming) and to a second set of plants during a stage when there was relatively no net synthesis of lipid (about six weeks after blooming). During both of these stages there was a net synthesis of protein. The leaves immediately below the panicle of the main stock were removed and the stem surface was bruised. An absorbent cotton wad was wrapped around the bruised area and a strip of scotch tape was placed

around the cotton. It was sealed tightly. Labelled acetate was injected through a hole in the tape with a hypodermic syringe. Approximately 2 microcuries were administered to each plant. After the first administration, seed boll samples were collected at 6, 12, 24 and 48 hours. After the second administration samples were collected at 1, 2, 3, 4 and 8 days.

The oil was extracted from each of the samples by methods already described and the lipid material was methylated. These methyl esters were separated by GLC and samples of the effluent gas were collected. The effluent gas was collected on 20 x 4 mm. filter paper strips impregnated with mineral oil. These strips were slipped into glass tubes 7 cm. long and 0.5 cm. in diameter which were placed at the outlet end of the gas chromatography column and exchanged at 30-second intervals till all the component fatty acids had passed through the column. These tubes were then held vertically in place over planchets and the mineral oil containing the fatty acids was washed onto the planchets with three 0.5 c.c. portions of petroleum ether. After the petroleum ether had evaporated, the material on the planchets was counted.

The crude material left after lipid extraction was hydrolyzed and the amino acids were separated on the Amino Acid Analyzer. The effluent solution was collected on a fraction collector in four minute fractions. Fractions from each individual acid were grouped together. Aliquots of the acids were plated for counting.

Counting was done on a gas flow detector equipped with a decade scaler, an automatic sample changer and a time interval printer. The

data were converted to counts per minute. The label in the fatty acids fractions was plotted and the areas under peaks were measured in order to find the activity in the fatty acids.

RESULTS AND DISCUSSION

Preliminary experiments to verify techniques

--Detection of methyl esters by GLC

To check the quantitative separation and detection by the 'Aerograph' a standard mixture of fatty acid methyl esters, distributed by the United States National Institute of Health (Metabolism study section standard D), was injected. In the following tabulation the data in columns 1 and 2 were provided by the supplier. Column 1 represents a shorthand designation of the structure of each fatty acid methyl ester in the mixture, i.e. 14:0 = a C_{14} acid with no double bonds. Column 3 is the data from the 'Aerograph.'

(1) methyl ester	(2) % by weight	(3) % by area from 'Aerograph'
14:0	11.82	13.4
16:0	23.61	24.9
16:1	6.84	8.0
18:0	13.08	11.1
18:1	44.62	42.6

The per cent by area data obtained from the 'Aerograph' was within two per cent of the actual per cent by weight composition of the mixture. This is within the acceptable limits recommended by USNIH (147). Therefore in all cases the per cent area values of the 'Aerograph' were assumed to be per cent by weight of the methyl ester samples analyzed.

--Estimation of amide nitrogen

It is generally assumed that in hydrolyzed samples much of the amide nitrogen is detected as ammonia. To test this assumption, a sample of glutamine was hydrolyzed and analyzed on the Spinco Amino Acid Analyzer. The ratio of ammonia to glutamic acid was found to be 1.00 to 1.21.

If the assumption that the amide nitrogen is split off on hydrolysis as ammonia is correct, a sample of glutamine should yield equal molar quantities of glutamic acid and ammonia. Since the molar quantity of ammonia detected in the hydrolyzed glutamine sample was 80 per cent of the molar quantity of glutamic acid, it appears to underestimate the amide nitrogen.

--Application of acetate-1, 2-C^{14}

In a preliminary study to determine the amount of uptake and translocation of acetate by flax plants, acetate 1, 2-C^{14} was administered in the described method, near the base of flax stems. Sixteen hours later the stems were cut into one-inch sections and the activity in each section was determined. The dosages applied and the extent of injury to the plants were as follows:

1. two microcuries (m.c.); leaves removed at area of application
2. four m.c.; leaves removed at area of application
3. four m.c.; leaves bruised but not removed
4. four m.c.; leaves removed and stem bruised.

The results are as follows:

	Counts per Minute			
Inches below tip	(1)	(2)	(3)	(4)
tip (includes bolls)	217	227	323	769
2	213	312	312	526
3	170	263	256	417
4	143	170	196	435
5	250	139	233	420
6	402	213	270	414
7	556	294	506	1428
8	558	509	503	1006
9	area of application	556	551	2513
10	238	area of application	714	3326
11	36		area of application	area of application
12			1111	560
13			119	101

Although (2) received twice as much label as (1), the amount translocated was about equal in these. However (4), which received the same concentration of label as (2) and (3), exhibited the highest rate of translocation. As a consequence 2 m.c. per plant were applied by method (4).

--Elution of labelled fatty acids from collector strips

The labelled fatty acids separated by GLC were eluted from the paper strip collectors onto planchets as already described. The following test was carried out to check the recovery of label by this elution method. Collector strips were washed successively with nine washes of petroleum ether. Groups of three washes were plated together.

	Counts per minute	
	Expt. 1	Expt. 2
1st three washes	316	133
2nd three washes	49	29
3rd three washes	17	21
background	18	18

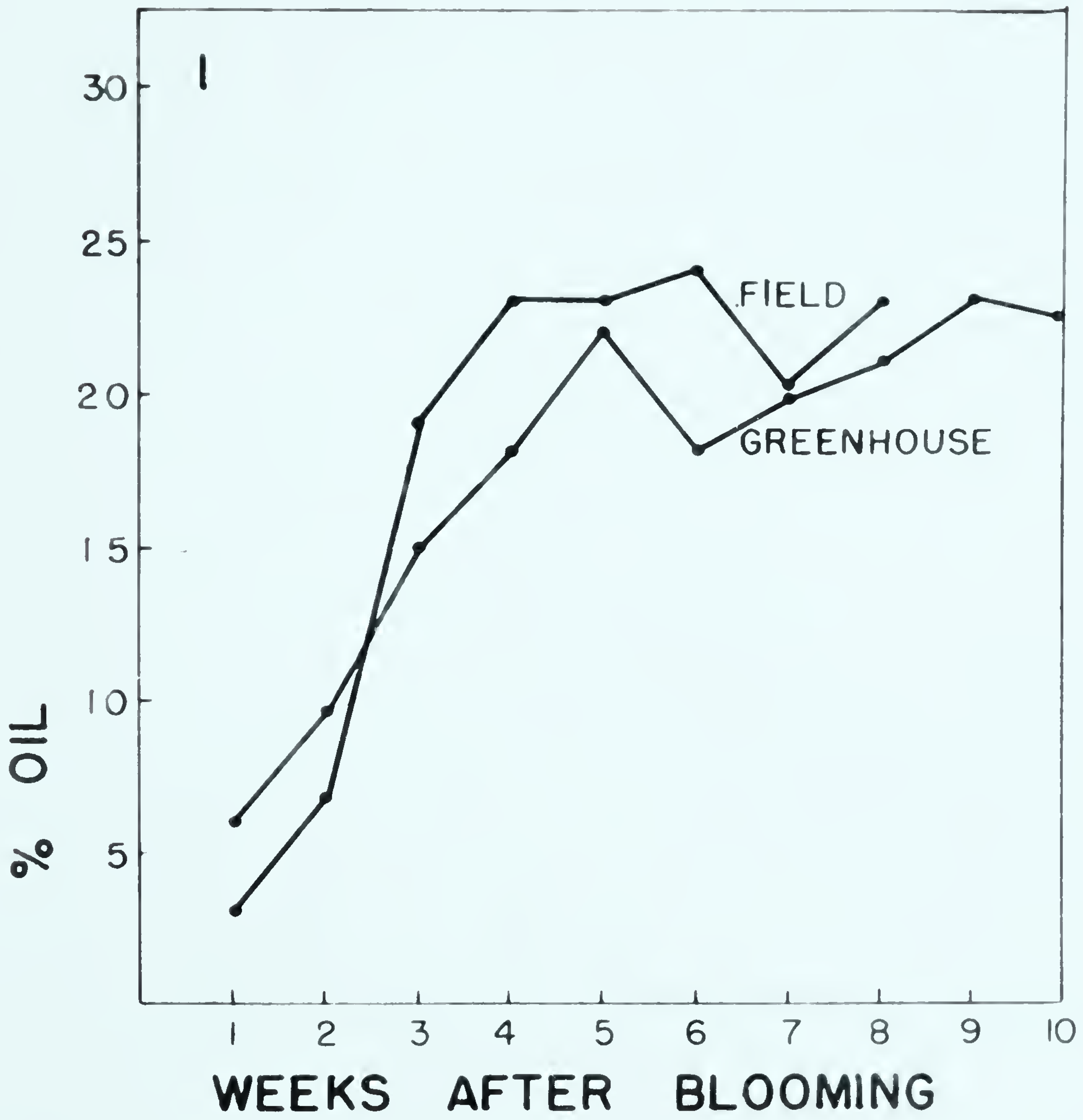
Since the label was almost completely washed out by the first three washings of the collector strip, all samples were eluted with three washings.

Oil and fatty acids in developing seeds

The per cent oil in developing flax bolls of both field and greenhouse grown plants are given in Figure 1. In both cases the oil content rose rapidly at first and then leveled off. The lipid content of the flax seeds themselves six and seven weeks after blooming was found to be between five and ten per cent higher than that of the intact bolls.

The oil content curves obtained in these developing seeds are typical of those reported by others for various oil plants. Oil deposition seems to take place mainly during the first four or five weeks after blooming.

FIGURE 1. Changes in per cent lipid content during development of flax.


1
% OIL
30
25
20
15
10
5
FIELD
GREENHOUSE
1
2
3
4
5
6
7
8
9
10
WEEKS AFTER BLOOMING

Painter (44) reports that oil deposition in the flax he studied began about the eighth day after flowering and was essentially complete in three weeks. Similarly in cotton (148) and in soybean (56) oil formation, although beginning somewhat later, took place over a period of only three weeks. In sunflower, however, (57) the oil content continued to increase for seven weeks.

The seed which was planted had an oil content of approximately 40 per cent. However the seed bolls at all stages of maturity investigated in this study were found to contain not more than 25 per cent lipid (Figure 1). As reported, the seeds themselves had somewhat more oil, but this still did not account for the differences in oil content observed. The conditions under which these plants developed were probably quite different from those for the parent seed. Temperature, light moisture and other factors which are inherently different under different growing situations could account for the differences in oil content. It is surprising, however, that plants grown in the field and those grown in the greenhouse, where environmental and other factors are so vastly different, did not display a great difference in oil content (Figure 1). A possible explanation is that field samples at eight weeks and greenhouse samples at ten weeks when collection of bolls was stopped may not have reached full maturity. However the oil content of the flax did not seem to increase significantly beyond four or five weeks after blooming. The seed of both the field and the greenhouse flax seemed by outward appearance to be mature at seven or eight weeks.

The fatty acid composition of the oil changed quite markedly in the second and third weeks after blooming (Figures 2 and 3). With the data plotted in this way it seemed that linolenic acid was being formed at the expense of other fatty acids. When the data were replotted as in Figures 4 and 5, it was apparent that this was not necessarily the case. On a dry weight basis a number of the fatty acids increased in concentration with time. Similar changes in fatty acid composition during flaxseed development have been reported (44, 54). However in oil palm (1) and in soybeans (56) an increase in saturation, not unsaturation, was noted during maturation.

The amounts of each acid in the oil increased quite rapidly during the first four or five weeks of development, after which the levels remained constant except in the greenhouse grown flax. Here a drop in linolenic acid content with a seemingly proportional rise in oleic acid content occurred after the fifth week (Figure 5). This suggested that oleic acid was being formed at the expense of linolenic acid. Hopkins and Chisholm (57) reported an increase in the weight of each fatty acid in developing sunflower and then at maturity a levelling effect. They found a decrease in the accumulation rate of oleic acid during rapid anabolism of oil with a concurrent increase in the rate of linoleic acid accumulation. They stated that this may be an indication that oleic acid was a precursor in the formation of linoleic acid.

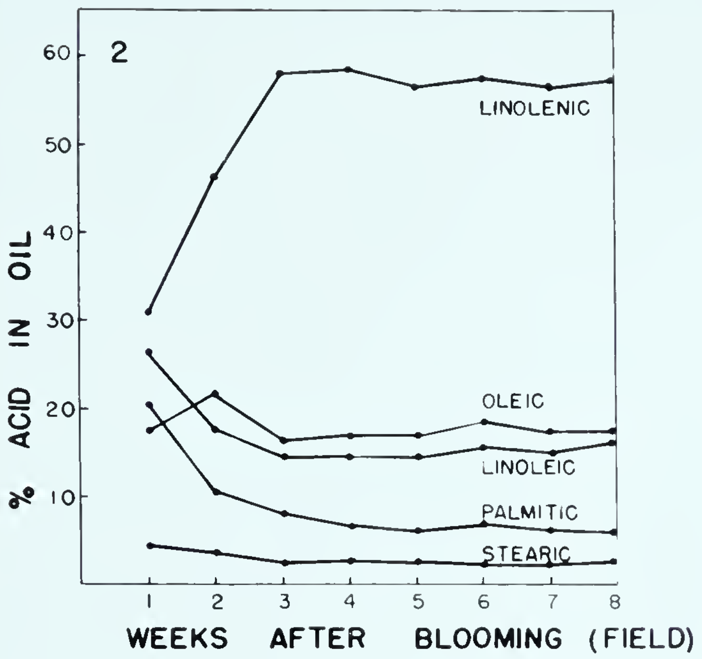
The fatty acid composition of mature field grown and of mature greenhouse grown flax was quite different (Figures 4 and 5). The oil from mature field grown flax had much more linolenic, somewhat less linoleic

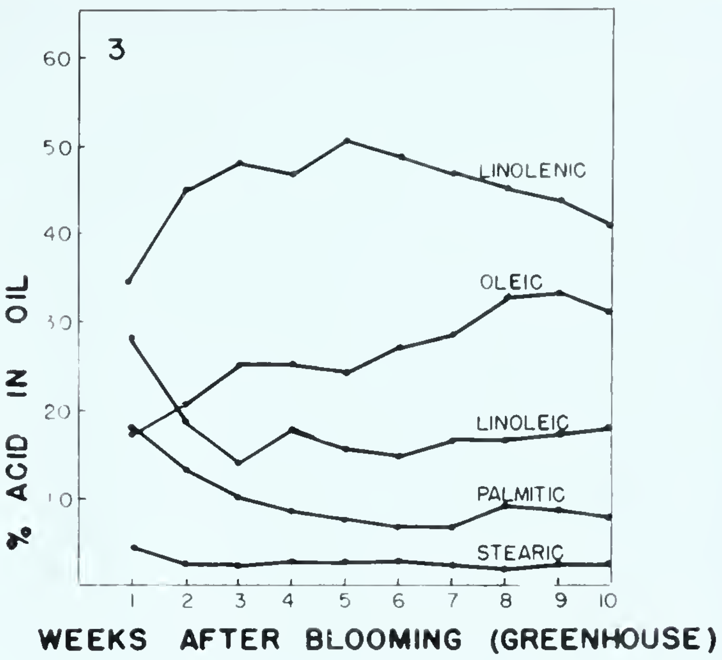
FIGURE 2. Changes in fatty acid composition during development of flaxseed in field expressed as percentage of total oil.

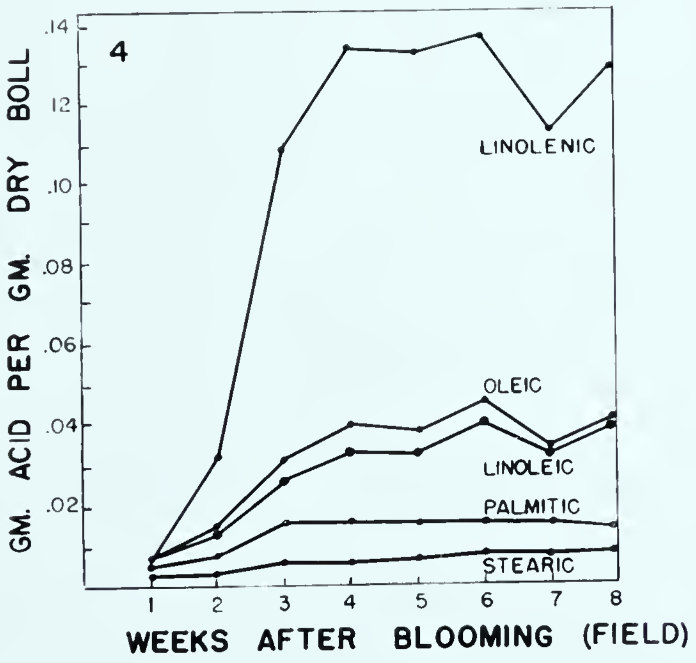
FIGURE 3. Changes in fatty acid composition during development of flaxseed in greenhouse expressed as percentage of total oil.

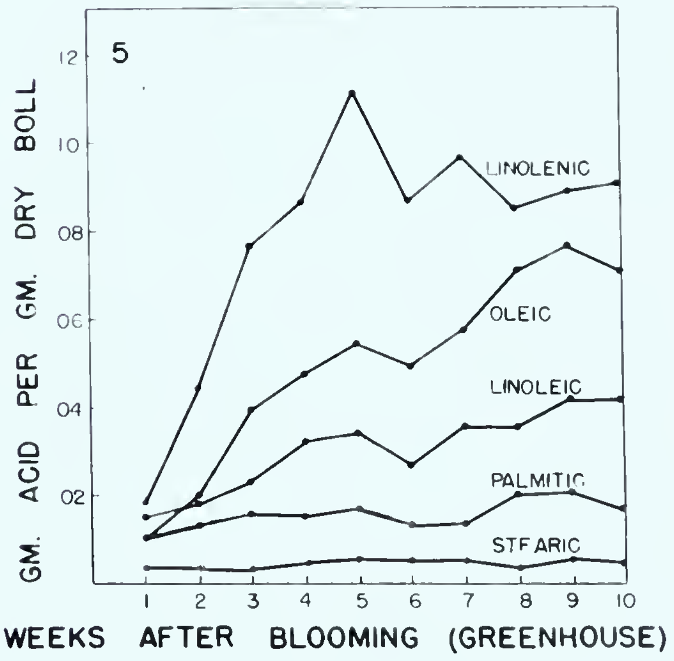
FIGURE 4. Changes in fatty acid composition during development of flaxseed in field expressed as weight of acid per gram dry boll weight.

FIGURE 5. Changes in fatty acid composition during development of flaxseed in greenhouse expressed as weight of acid per gram dry boll weight.


2
% ACID IN OIL
60
50
40
30
20
10
LINOLENIC
OLEIC
LINOLEIC
PALMITIC
STEARIC
1 2 3 4 5 6 7 8
WEEKS AFTER BLOOMING (FIELD)


3
% ACID IN OIL
60
50
40
30
20
10
LINOLENIC
OLEIC
LINOLEIC
PALMITIC
STEARIC
1 2 3 4 5 6 7 8 9 10
WEEKS AFTER BLOOMING (GREENHOUSE)


4
GM. ACID PER GM. DRY BOLL
.14
.12
.10
.08
.06
.04
.02
LINOLENIC
OLEIC
LINOLEIC
PALMITIC
STEARIC
1 2 3 4 5 6 7 8
WEEKS AFTER BLOOMING (FIELD)


5
GM. ACID PER GM. DRY BOLL
.12
.10
.08
.06
.04
.02
LINOLENIC
OLEIC
LINOLEIC
PALMITIC
STEARIC
1 2 3 4 5 6 7 8 9 10
WEEKS AFTER BLOOMING (GREENHOUSE)

and much less oleic acid than the oil of greenhouse grown flax. The saturated acid composition was alike in the two lots of mature oil. Thus the field flax had a more unsaturated nature. This may have been due to a difference in temperature during maturation of the two groups of plants. The temperature in the greenhouse was kept at 21°C. The average temperature in June, July and August in 1961 when the field grown flax seed was maturing was approximately 18° C.* Therefore the oil of flax matured under warmer greenhouse conditions was more saturated. Similar findings of greater saturation in the oil of oilplants grown under warm temperatures than in oil of those grown under cool temperatures were also reported by Hilditch (42), Barker and Hilditch (47), Grindley (49) and by McGregor and Carson (50). All of these workers assumed that the differences found were due to temperature alone. It seems probable, however, that the differences may have been due to other environmental factors. Conditions such as different light periods, different light intensities, different moisture reserves or nutrition reserves and so forth are possible reasons for the observed differences. For example, the daily light period at Fort Vermillion, Alberta where a less saturated flaxseed oil was produced is longer than at Morden, Manitoba (50) and thus may have influenced the amount of saturation in the oil. In order to state that the differences observed were due to temperature alone, experiments with better control of all the variables would be required. All of the above experiments were conducted on field plots where it was impossible to keep environmental

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* Climatologist, Dominion Meteorlogical Station, Edmonton.

conditions constant. Plessers (149) reported marked morphological differences in greenhouse experiments with flax under different light, temperature and moisture conditions, but unfortunately he did not report on differences in chemical constitution.

As already reported, there was a difference of only 3°C in the average temperature of the field and greenhouses. As the environment during maturation in the greenhouse and in the field was no doubt vastly different, it is quite evident that one or several environmental factors played a distinctive role in determining the final oil composition.

Hilditch (51) reviewed aspects of oil synthesis at different temperatures. He assumed that oil formed at a cooler temperature was not as complete as that formed at a higher temperature. Because the oil was more saturated when formed at a high temperature than when formed at a low temperature, he concluded that more saturated fatty acids are formed from less saturated ones. Hilditch's assumption that a more complete oil is formed at a high temperature than at a low temperature may, however, not be plausible. In the present study the fatty acid composition of the oils did not change materially from the fifth week after blooming, but the fatty acid complements were distinctly different for the two environments beyond this stage. It seems, therefore, that the oil under a fixed set of conditions has a definite mature composition. If this is correct, it can be stated that each oil is complete, regardless of temperature or other factors. Probably for any set of environmental conditions the action of enzymes or other controlling mechanisms is such that only a certain fatty acid complement is formed.

Therefore it may be misleading to draw conclusions about fatty acid synthetic pathways from compositional differences of mature oils.

The saturated fatty acid composition was found to be similar in the oil of greenhouse and field grown flax. Similarly, other workers (50, 42, 49) reported saturated fatty acid compositions to be the same for plants grown under different environmental conditions. It appears that the formation of saturated fatty acids is not as sensitive to environment as the formation of unsaturated fatty acids.

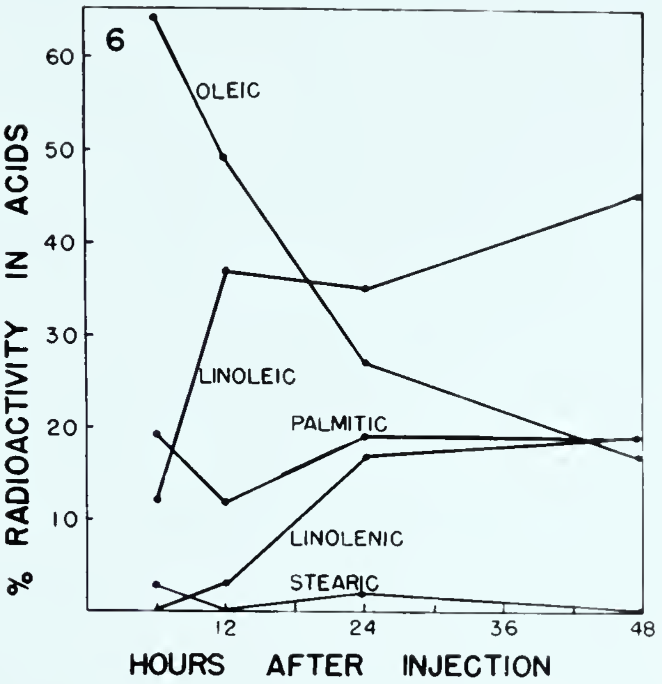
Radioactive isotope studies were carried out on developing seeds at a stage approximately one week after blooming and at a stage about five weeks after blooming. The results of the first stage are illustrated in Figures 6 and 7. It can be seen that the percentage of activity in oleic acid was very high at first (Figure 6) but the activity fell steadily. The percentage activity in linoleic acid rose rapidly in the first 12 hours, and the percentage activity in linolenic acid rose fairly fast in the first 24 hours after injection. The saturated fatty acids remained quite constant with respect to the amount of activity they contained. In Figure 7 the data are represented as counts per minute per microlitre methyl ester. The counts per minute in linoleic acid per microlitre methyl ester increased most rapidly. Oleic acid in proportion to the rest increased in activity very slowly and stearic did not increase in activity at all. The label in palmitic and linolenic acid did not increase in the first twelve hours.

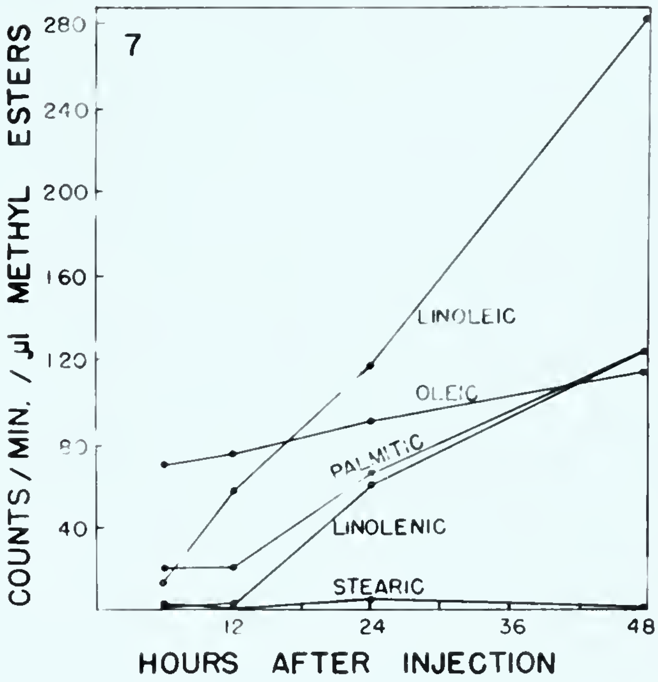
FIGURE 6. Percentage of total lipid label in various fatty acids after acetate 1, 2-C^{14} administration to flaxseeds at early stage of development.

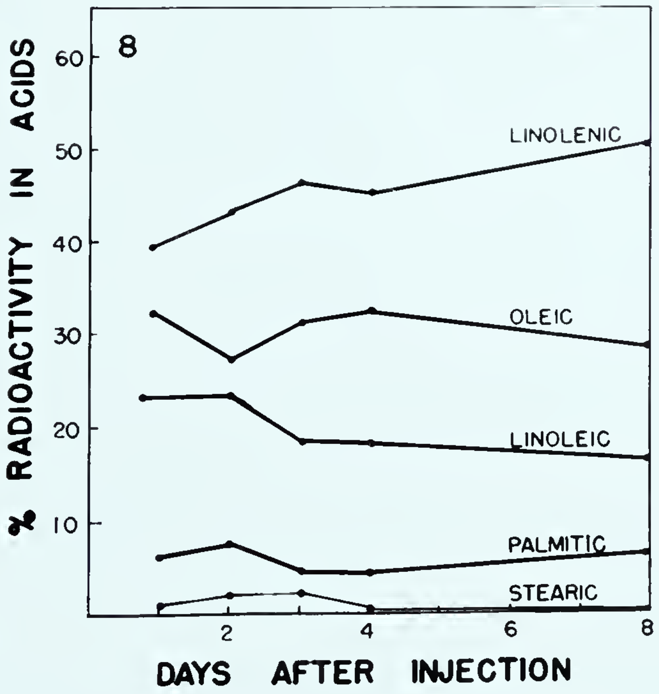
FIGURE 7. Counts per minute per microlitre methyl ester (i.e. total lipid) in various fatty acids after acetate 1, 2-C^{14} administration to flaxseeds at early stage of development.

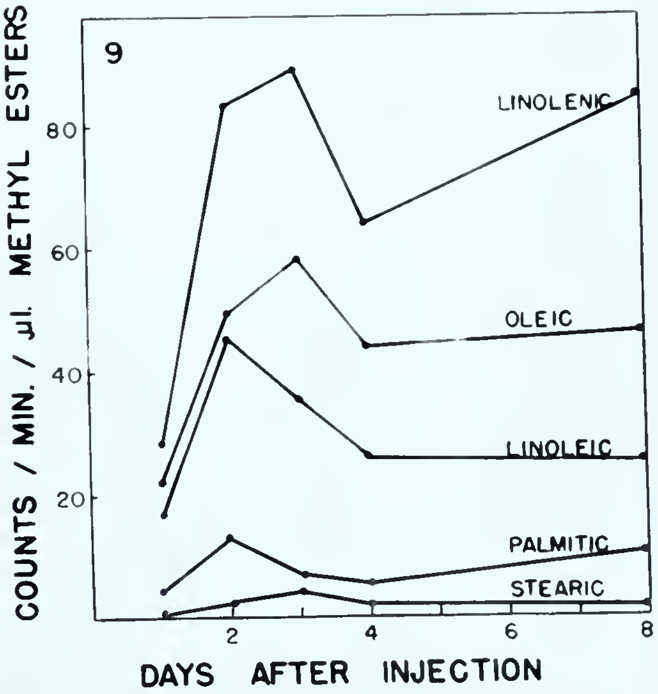
FIGURE 8. Percentage of total lipid label in various fatty acids after acetate 1, 2-C^{14} administration to flaxseeds at late stage of development.

FIGURE 9. Counts per minute per microlitre methyl ester (i.e. total lipid) in various fatty acids after acetate 1, 2-C^{14} administration at late stage of development.


6
OLEIC
LINOLEIC
PALMITIC
LINOLENIC
STEARIC
% RADIOACTIVITY IN ACIDS
10
20
30
40
50
60
12
24
36
48
HOURS AFTER INJECTION


7
LINOLEIC
OLEIC
PALMITIC
LINOLENIC
STEARIC
COUNTS / MIN. / µl METHYL ESTERS
40
80
120
160
200
240
280
12
24
36
48
HOURS AFTER INJECTION


8
LINOLENIC
OLEIC
LINOLEIC
PALMITIC
STEARIC
% RADIOACTIVITY IN ACIDS
10
20
30
40
50
60
2
4
6
8
DAYS AFTER INJECTION


9
LINOLENIC
OLEIC
LINOLEIC
PALMITIC
STEARIC
COUNTS / MIN. / µl. METHYL ESTERS
20
40
60
80
2
4
6
8
DAYS AFTER INJECTION

These radioisotope studies indicate that neither stearic nor palmitic acids are precursors of oleic acid, but that oleic acid may be a precursor of linoleic, linolenic and palmitic. In Figure 6 the percentage of the total activity which is concentrated in oleic acid is quite high, but this falls drastically while the percentage of activity in linoleic acid increases. This can be taken as evidence that linoleic acid is being formed from oleic acid, a conclusion also reached by James (150) and by Simmons and Quackenbush (60) in similar studies. This agrees with the pathway of linoleic acid synthesis described by Ching Yaun and Bloch (61) in yeast. In Figure 6 it is suggested that linoleic acid is a precursor of linolenic acid. After 12 hours of label administration the percentage of activity in linoleic acid does not increase greatly, while the percentage of activity in linolenic acid does.

In Figure 7 there is again a strong indication that linoleic acid is being preferentially formed from oleic acid. It appears that linolenic acid and palmitic acid, which later increase quite rapidly in counts, are also formed from oleic acid. Previous to the first analysis (i.e. at six hours), oleic acid increased rapidly in the amount of label it contained. Linoleic acid began forming rapidly about six hours, and oleic acid was probably being reconverted to linoleic, the activity in oleic remaining low. The activity of linoleic acid increased constantly even after twelve hours when linolenic acid began to increase rapidly in activity. The linoleic acid may have been formed at such a rate that the drain on linoleic, a possible precursor of linolenic acid (62), was not noticeable. It is of interest to note that the activity rise in palmitic

acid almost parallelled the rise in linolenic acid activity. This suggests a common precursor, but may have been the result of separate pathways operating at the same rate.

Following the later stage of injection the percentage of radioactivity in the acids remained approximately constant throughout the period of observation (Figure 8). In Figure 9 all acids fluctuated more or less in rhythm with each other. The amount of label taken up at this stage was considerably less than that taken up at the earlier stage of acetate administration.

It appears from Figures 8 and 9 that at the later stage the labelled acetate taken up was not used in net synthesis of lipid. An equilibrium between synthesis and breakdown of the lipid had been established so that there was no net synthesis or net breakdown. Therefore the percentage of the total activity contained by any one acid would be expected to correspond to the percentage of the total lipid represented by that acid. This was found to be the case in this study (Figures 3 and 8). Whether or not complete breakdown and complete synthesis of fatty acids occurred during the state of dynamic equilibrium was not too well established. There is some evidence that fatty acids during dynamic equilibrium are synthesized completely by pathways similar to those of net synthesis (6). If this were so, the labelling pattern following acetate injection at a state of equilibrium should have been similar to the pattern during net synthesis. The first samples analyzed from the later radioisotope study were taken one day after label administration. The labelling pattern may have been similar to that of the early study, but after one day at the

later stage the labelling pattern was much different from that after 24 hours at the early stage. On the basis of these results it is doubtful that the acids during the equilibrium state were completely broken down and resynthesized. Instead the acids may have been partially broken down by α or β-oxidation and then once again been built up. In this way labelled acetate may have been taken up without displaying the label pattern of the studies at the earlier stage.

The label taken up at the later stage was less than that taken up in the earlier stage (Figures 7 and 9). This indicated that any synthesis which took place during the state of equilibrium was much less extensive than that during net synthesis.

Between the third and fourth day after injection of label into the five-week flax, all of the acids dropped in radioactivity. This may have been due to drying of the cotton wad by which the label was applied and thus no labelled acetate was taken up. Some of the acids were probably broken down and the radioactive carbon may have been respired or replaced by normal carbon. The cotton wad was wetted with demineralized distilled water at four days. The activity of the various acids then levelled off, indicating that acetate was again being incorporated. Simmons and Quackenbush (60) with soybeans found that when radioactive sucrose substrate was replaced by non-labelled sucrose the acids continued to increase in label content, probably due to the storage in the plant cells of radioactive sucrose or some other metabolite formed from sucrose. Presumably this was not the case when radioactive acetate was the substrate in developing flax.

Oil and fatty acids in germinating seedlings

The total oil content of flax seedlings in the light and in the dark at each of the seven days of germination is shown in Figure 10. For the first three days in both the light and dark, the oil content remained at approximately the level it was in the seed. After this the oil content fell sharply till about six or seven days when it levelled off.

The shape of lipid percentage curves during the germination of oil-crops has been reported countless times. The curves found in this study are typical. A rise in carbohydrate content corresponding to the fall in lipid constitution is also common (99). Although it was commonly accepted that the fat was converted to carbohydrate, the glyoxylate cycle which explains this interconversion was not described until 1957 (5). No determinations of carbohydrate were made in these flax studies and no studies to check the operation of the glyoxylate cycle were carried out. However, since flax is a crop rich in oil and since it displays typical curves of oil consumption, it can be stated with reasonable assurance that a conversion of fat to carbohydrate explains the fall in fat content.

Since the two curves in Figure 10 are almost duplicate, light probably has little effect on the operation of the glyoxylate cycle.

During the seven days of germination there were surprisingly few changes in fatty acid composition. Figures 11 and 12 illustrate the small amount of variation in percentage fatty acid and it is shown in Figures 13 and 14 that during the stage of rapid lipid breakdown the acids are utilized more or less in proportion to the amounts originally present in the seed.

FIGURE 10. Changes in per cent lipid content of flax during germination.

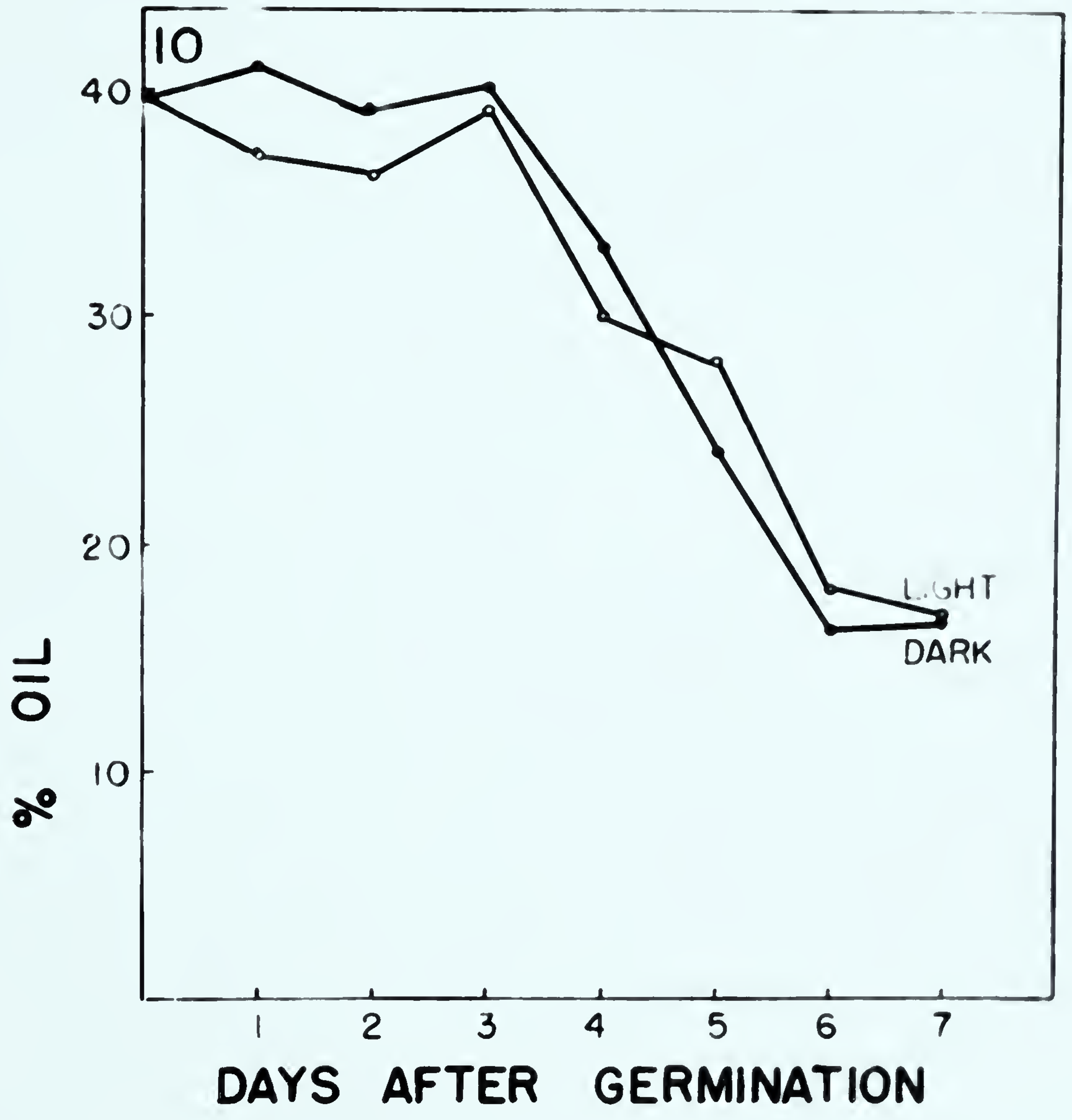
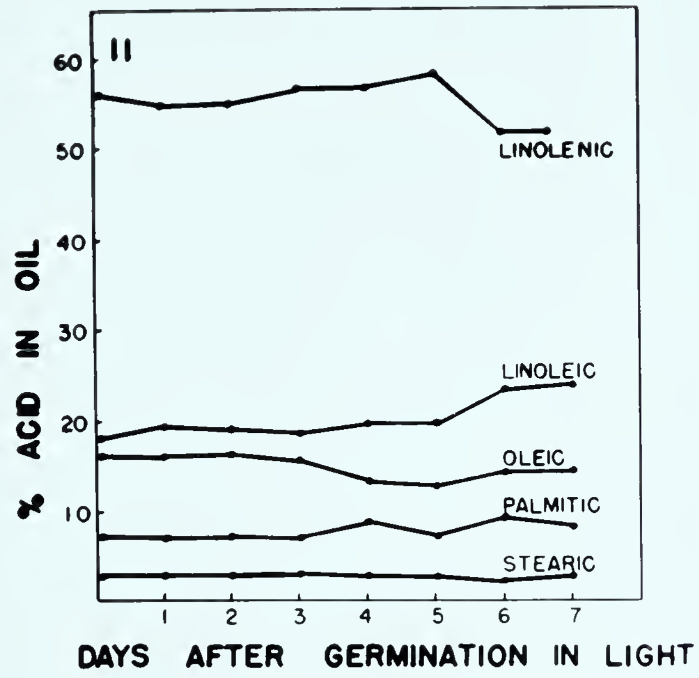

10
% OIL
40
30
20
10
1
2
3
4
5
6
7
DAYS AFTER GERMINATION
LIGHT
DARK

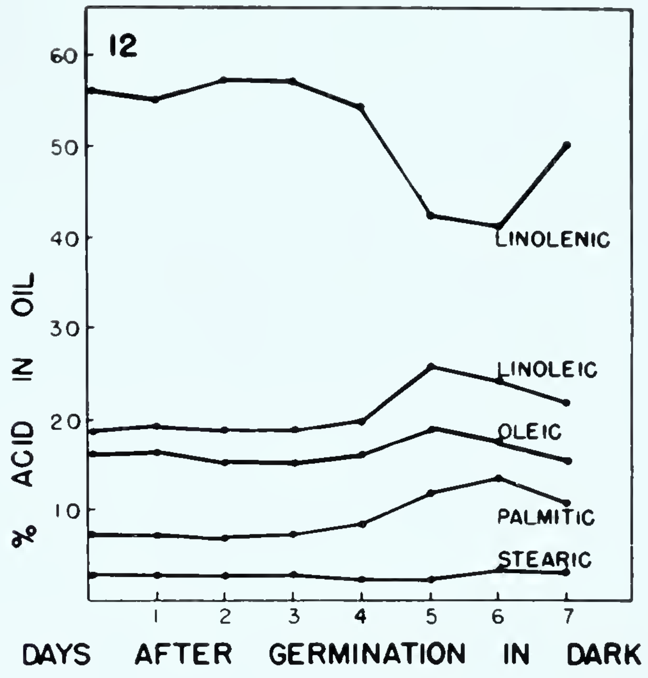
FIGURE 11. Changes in fatty acid composition during germination of flax in the light expressed as percentage of total oil.

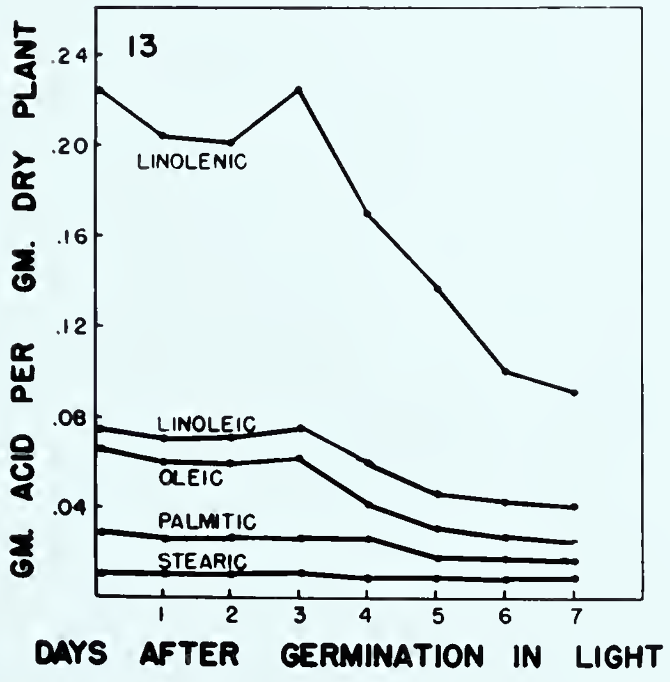
FIGURE 12. Changes in fatty acid composition during germination of flax in the dark expressed as percentage of total oil.

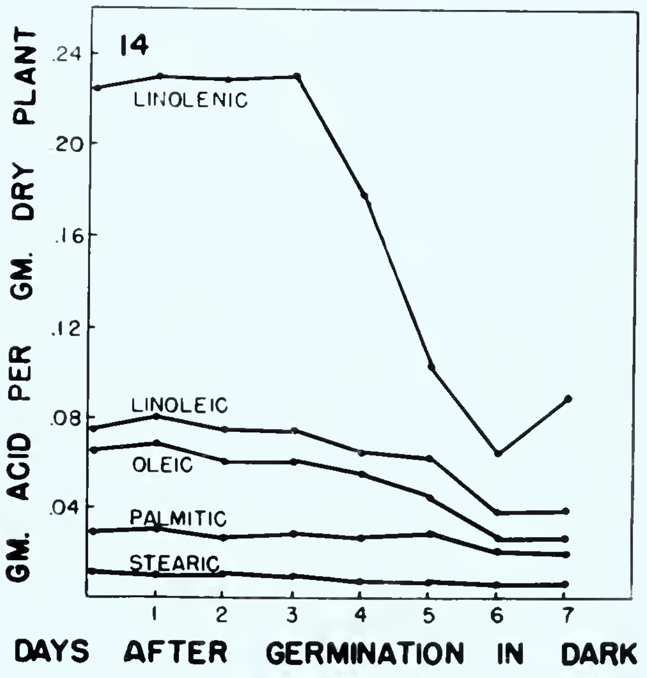
FIGURE 13. Changes in fatty acid composition during germination of flax in the light expressed as weight of acid per gram dry plant weight.

FIGURE 14. Changes in fatty acid composition during germination of flax in the dark expressed as weight of acid per gram dry plant weight.


11
60
50
40
30
20
10
% ACID IN OIL
LINOLENIC
LINOLEIC
OLEIC
PALMITIC
STEARIC
1
2
3
4
5
6
7
DAYS AFTER GERMINATION IN LIGHT


12
60
50
40
30
20
10
% ACID IN OIL
LINOLENIC
LINOLEIC
OLEIC
PALMITIC
STEARIC
1
2
3
4
5
6
7
DAYS AFTER GERMINATION IN DARK


13
.24
.20
.16
.12
.08
.04
GM. ACID PER GM. DRY PLANT
LINOLENIC
LINOLEIC
OLEIC
PALMITIC
STEARIC
1
2
3
4
5
6
7
DAYS AFTER GERMINATION IN LIGHT


14
.24
20
.16
.12
.08
.04
GM. ACID PER GM. DRY PLANT
LINOLENIC
LINOLEIC
OLEIC
PALMITIC
STEARIC
1
2
3
4
5
6
7
DAYS AFTER GERMINATION IN DARK

The results of the determinations of fatty acid composition during germination indicate that the enzymes of the α and β-oxidative chains are non-preferential in their action invivo. Had the action of the enzymes involved been preferential, the acid composition during germination would have changed considerably. Several studies have indicated that α and β-oxidation take place immediately after hydrolysis by lipase (70, 74, 76). If this is true, it can be assumed that lipase activity is also nonpreferential during rapid lipid breakdown. However if oxidation does not take place immediately after the hydrolytic splitting of the triglyceride molecule, acids could be split off to form free fatty acids or other derivatives which may accumulate before oxidation. This splitting off could occur in a selective manner but if oxidation did not occur selectively the overall fatty acid composition would remain constant.

Other studies on fatty acid composition during germination in general agree with the results of this study (72, 73, 74, 76). Some results, however, are in slight disagreement with the above. Rabari et al (75) report that saturated fatty acids are used more rapidly than unsaturated fatty acids during the germination of peanuts. Oleic acid was found to be preferentially utilized in germinating watermelon seedlings by Crombie and Comber (71). After the breakdown period they noted the formation of small quantities of an oil with a large proportion of linolenic acid. They thought that this synthesis was associated with photosynthesis.

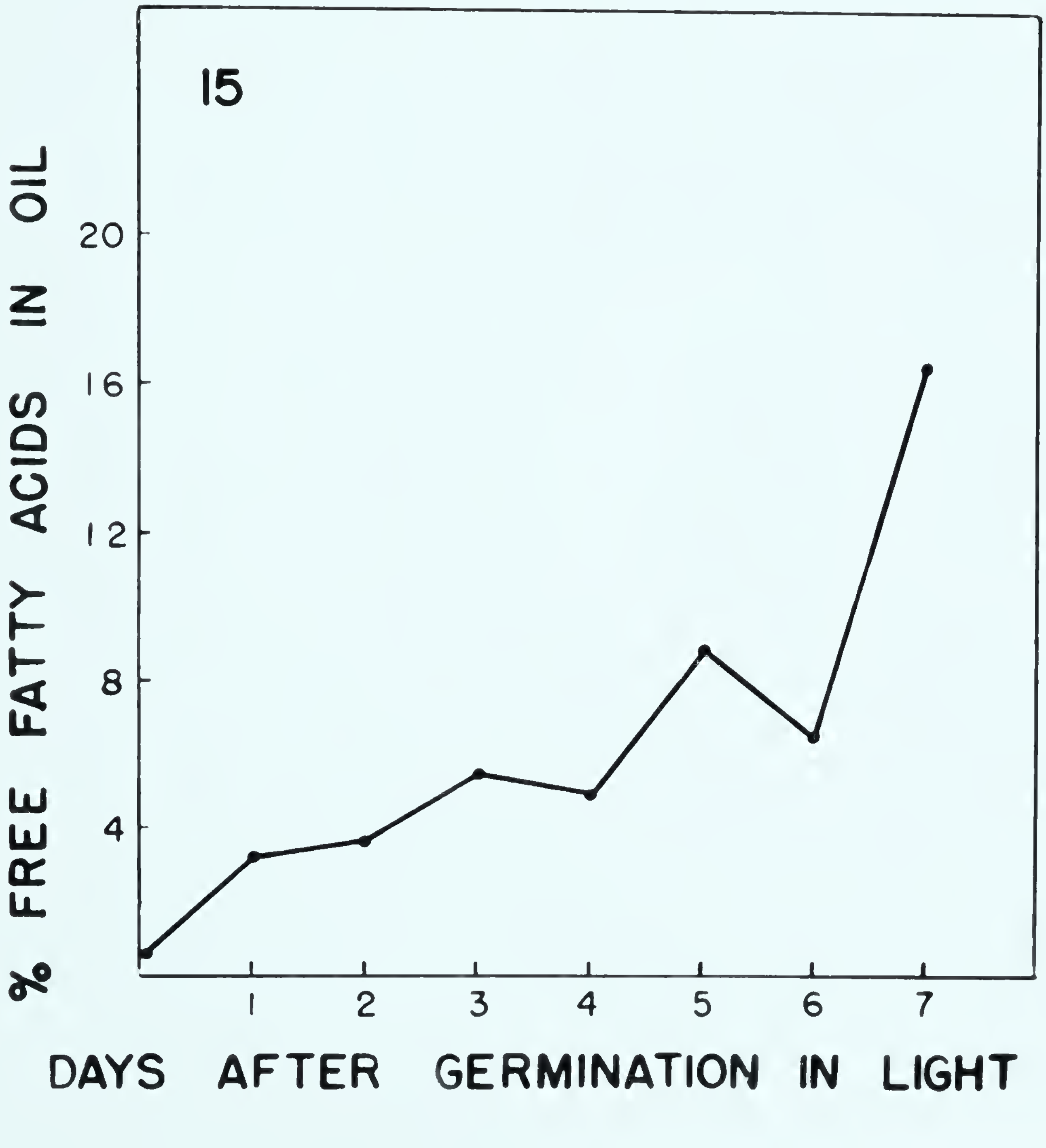
In this study the fatty acids were metabolized similarly in the dark and in the light. Thus an oil associated with photosynthesis was probably

not formed during the germination of flax. White (73), working with light and dark germinated cottonseeds, also found no apparent difference in the oil composition as a result of illumination differences.

The methylation procedure of Craig and Murty (142), using sodium as catalyst, is considered to be specific for pure triglycerides. It was found not to methylate the fatty acids of lipid after four days of germination. Therefore they could not be detected by GLC. However, the Metcalfe and Schmitz (143) method using BF_3, which is believed specific for free fatty acids, was satisfactory in methylating these lipids. The fact that the first method will not methylate free fatty acids and that the BF_3 method will not affect the methylation reaction in neutral oils was established in preliminary work. A study was thus conducted to determine the free fatty acid content of the oil during germination. As seen in Figure 15, the free fatty acid content rose slowly to about 16 per cent of the oil.

The 16 per cent free fatty acid content of the oil during germination (Figure 15) is somewhat greater than the usual values reported but it still does not explain the fact that until four days of germination the oil is easily methylated by the Craig and Murty method but following this the method of Metcalfe and Schmitz must be applied to obtain GLC detectable quantities of methyl esters. Thus the lipid material present cannot be accounted for by neutral oil and free fatty acids alone. If the lipid not present as free fatty acid had all been neutral oil it would have been detected by GLC when methylated by the Craig and Murty method. Because after four days of germination the lipid is not detected when

FIGURE 15. Free fatty acid content of flax oil during germination in the light.


15
% FREE FATTY ACIDS IN OIL
20
16
12
8
4
1
2
3
4
5
6
7
DAYS AFTER GERMINATION IN LIGHT

only methylated by this method, the neutral oil probably did not constitute as much of the lipid as might be assumed. Likewise the free fatty acid content at any time was not high and it is therefore difficult to explain the ability of BF_3 to methylate detectable quantities of the lipid fraction after four days. Probably BF_3 catalyzes the methylation of some fatty acid derivative. The most likely derivatives of fatty acids, which might be methylated with BF_3, are the CoA derivatives. These would escape detection by the A.O.C.S. method (141) for free fatty acids (NaOH titration). If these CoA or other derivatives were methylated by BF_3, it would provide a logical explanation for the results obtained as acyl CoA molecules are absolutely essential to β-oxidation, the process which is supposedly occurring at the time of rapid oil breakdown. If this is the explanation for the observed results, it follows that immediately after lipase hydrolysis most of the fatty acid molecules undergo the thiokinase reaction of β-oxidation. This converts them to their CoA derivatives and they are no longer detectable as free fatty acids. No attempt was made to investigate this hypothesis.

No short chain fatty acids or their derivatives were detected at any stage in this study. The methods of methylation used were such that short chain acids could easily have been lost by volatilization. There are no reports of an accumulation of lower chain fatty acids during the period of fat breakdown in germinating seeds (11).

Protein and amino acids

The protein content of germinating flax seedlings (Figure 16) remains almost constant in light and in darkness. The protein content was based

on total nitrogen X6.25. However, observing the free amino acid curve, one can note that as germination proceeded, more and more of the nitrogen was present as free amino acids.

An amino acid analysis was performed on the protein after one day and after seven days of germination. As illustrated in the following tabulation, the amino acid composition changed very little.

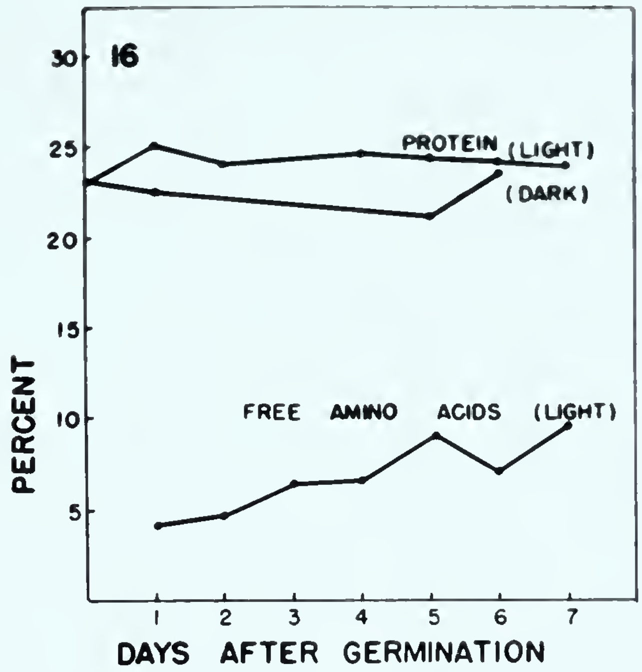
	1st day (micromoles)	7th day
glutamic acid	3.979	3.390
arginine	1.535	1.008
proline	1.014	1.428
aspartic acid	2.328	2.120
lysine	0.887	0.994
threonine	0.932	0.982
methionine	0.385	0.368
isoleucine	0.997	0.892
serine	1.284	1.439
glycine	2.445	2.640
alanine	1.505	1.694
valine	1.325	1.313
leucine	1.155	0.998
tyrosine	0.391	0.448
phenylalanine	0.855	0.717
histidine	0.404	0.479
ammonia	4.423	4.959

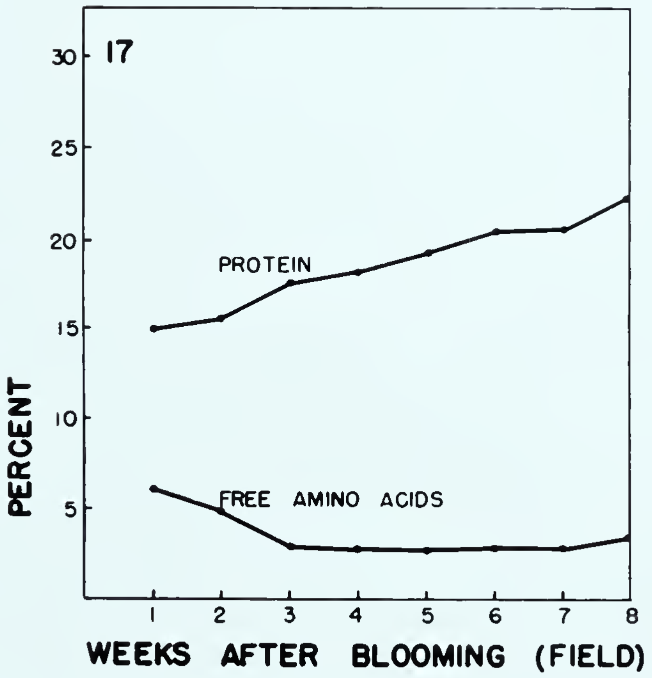
The constant protein levels and the constant amino acid makeup of the protein during germination were expected since protein is probably in a state of equilibrium at this point (132, 133). The proteins are probably not being used as energy at this stage but are probably being broken down to their component amino acids. These amino acids are presumably translocated later and utilized to form the protein of the hypocotyl.

FIGURE 16. Protein content and free amino acid content during germination of flax.

FIGURE 17. Changes in protein and free amino acid contents during flaxseed development in the field.

FIGURE 18. Amino acid family composition during field development of flaxseed.


16
PROTEIN (LIGHT)
(DARK)
FREE AMINO ACIDS (LIGHT)
PERCENT
30
25
20
15
10
5
1
2
3
4
5
6
7
DAYS AFTER GERMINATION


17
PROTEIN
FREE AMINO ACIDS
PERCENT
30
25
20
15
10
5
1
2
3
4
5
6
7
8
WEEKS AFTER BLOOMING (FIELD)

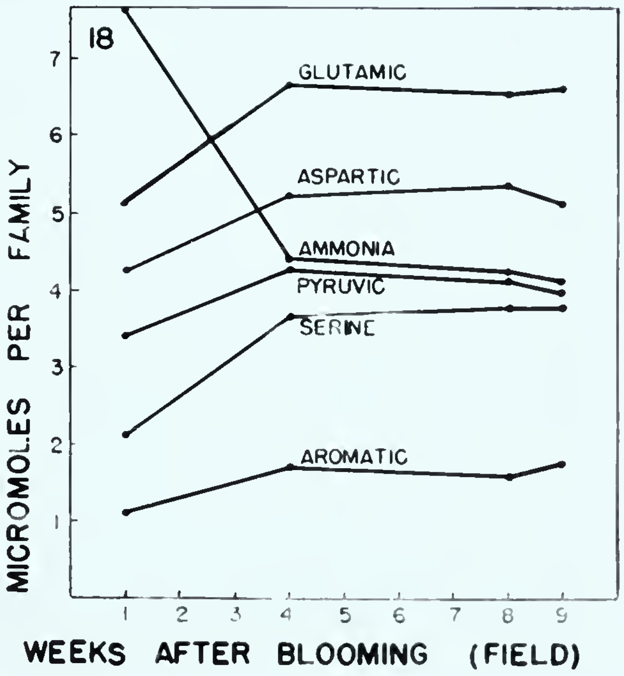

18
GLUTAMIC
ASPARTIC
AMMONIA
PYRUVIC
SERINE
AROMATIC
MICROMOLES PER FAMILY
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
WEEKS AFTER BLOOMING (FIELD)

Figure 17 gives a picture of the protein and free amino acid content of the developing flaxseed after blooming. The protein content rose steadily but the percentage of free amino acids dropped for the first three weeks and then remained fairly constant.

The amino acid composition by families of the developing seeds is shown in Figure 18. Although this grouping was done to make the graph less complicated, the individual members in general were found to mimic the behavior of the family as a whole. The level of ammonia seems to have fallen as the amount of amino acids increased.

Comparing Figures 17 and 18, it may be seen that as the protein and amino acid contents increased after blooming, the free amino acid and amide nitrogen (ammonia) fell. The fall in free amino acid content and in the amount of amide nitrogen was almost parallel. Thus the free amino acids may have been mainly in the amide form. Sampaio and Folkes (129) found that most of the amide in germinating barley seedlings was in the peptide fraction but Spragg (130) found free amino acids particularly high in amide content. It seems that the amide fraction plays an important role in flax during the stage of protein development. The amide nitrogen content of many plants seems to be of some importance (128, 129, 130, 131) in both protein synthesis and breakdown, however its exact role is not established. It may be a vehicle for nitrogen storage, nitrogen transfer, or for nitrogen translocation. Observation of Figure 18 reveals that the role of amides in developing flax is probably a means of storing nitrogen. As nitrogen is needed for the synthesis of amino acids, the amide nitrogen is depleted. When there is

no more net synthesis of amino acids, as observed from four weeks after blooming onwards, the amide nitrogen content is not decreased.

The results of isotopic amino acid studies during development were as follows:

		Counts per minute/micromole amino acid			
		6 hours	12 hours	24 hours	48 hours
glutamic family:	glutamic acid	27.6	73.5	181.3	149.0
	arginine	0.0	45.4	124.2	115.9
	proline	16.7	27.1	151.7	289.0
aspartic family:	aspartic acid	0.0	25.4	27.0	29.4
	lysine	5.4	33.8	40.4	43.9
	threonine	38.7	12.8	3.1	14.2
	methionine	40.7	0.0	0.0	9.7
	isoleucine	14.4	7.1	13.6	2.4
serine family:	serine	0.0	0.0	1.5	1.0
	glycine	7.4	95.4	0.0	0.0
pyruvic family:	alanine	1.3	48.7	0.0	5.1
	valine	36.6	0.0	0.0	1.4
	leucine	15.7	3.6	0.0	2.9
aromatic family:	tyrosine	80.0	54.5	0.0	0.0
	phenylalanine	0.0	0.0	0.0	6.3
	histidine	169.4	78.5	22.8	38.2

The members of the glutamic family increased in activity during the experiment and were generally the most highly labelled amino acids. The activity in aspartic acid and lysine rose simultaneously with a fall in the label of the other three members of the aspartic family. Serine did not become labelled while its partner, glycine, was highly labelled at 12 hours after injection. The labelling in the acids of the pyruvic family did not appear to follow any pattern. Tyrosine and histidine were heavily labelled after the first collection, but their amount of

label decreased with time after injection. Phenylalanine did not show any appreciable label.

The results of the amino acid tracer studies must be accepted as being of a preliminary nature. Because of limited time these amino acid analyses were not done in duplicate. Furthermore the low counts in the four minute aliquots which were collected forced the combining of these into fractions representing individual amino acids. Aliquots of the amino acid fractions were then plated for counting. The inevitable errors involved would be multiplied several times in calculating the count in an amino acid from the counts in the single aliquot and in calculating the counts per micromole amino acid detected, as in most cases there was much less than one micromole of amino acid.

Nevertheless these data gave some indication of what may have taken place. The extent of labelling in the glutamic family agreed with the results obtained by Bilinski and McConnell (127) in wheat. Results such as obtained by Roberts et al (125) or by Cowie and Walton (126) in which heads of families seemed to be precursors to the incorporation of C^{14} by other members were not obtained in this study except in the case of glutamic acid which seemed to become labelled faster at the beginning than the other two members of the family.

The labelling pattern in the aspartic family may indicate that threonine, methionine and isoleucine were precursors for aspartic acid and lysine.

There was no indication of an interconversion of glycine to serine (serine family) in this study. Such an interconversion was reported in

wheat by McConnell and Bilinski (151). The increase in amount of label at twelve hours in glycine and some of the other unexplainable variations may simply have been due to experimental errors.

One of the most surprising findings in the isotope study was the labelling pattern in tyrosine and histidine. At six hours after injection both were relatively highly labelled but the amount of label decreased with time. McCalla and Neish (152) reported the synthesis of phenylalanine and of tyrosine from shikimic acid. As carbohydrate is a precursor to shikimic acid, the acetate injected would first have to be converted to carbohydrate. Although this may have occurred, only the tyrosine contained label. There is no evidence of such a pathway in the formation of histidine. Neidle and Waelsch (153), who have reviewed the path of histidine formation, suggest it is formed by way of a purine ring.

Little evidence pertaining to 'primary' or 'secondary' origin of amino acids was gained from this study.

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